University of Alberta

Phylogenetic relationships of the Phocidae and population genetics of ice breeding seals

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Systematics and Evolution

Department of Biological Sciences

Edmonton, Alberta

Spring 2004

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Abstract

Due to their evolutionary origins among the terrestrial carnivores, the distribution of all pinnipeds (seals, sea lions, and walrus) is essentially costal or associated with ice in high latitudes. Although pinnipeds forage at sea and can maintain a pelagic existence for long periods of time, they must leave the water annually for parturition. Individuals are generally solitary and only interact socially during the highly synchronized pupping and mating season. The predictability and stability of different breeding habitats may result in varying levels of site fidelity that either promote or deter the development of population structure.

Breeding areas for species that give birth on land are easily identified, allowing for direct examinations of population structure using traditional methods such as mark-recapture and telemetry. In contrast, for many ice breeding species direct observation of movement and dispersal is not feasible due to the relative inaccessibility of their habitat. Therefore, microsatellite markers were used to investigate genetic variation and population structure in four species of Antarctic and two species of Arctic ice breeding seals.

Among the Antarctic ice breeding species examined, Ross (*Ommatophoca rossii*), leopard (*Hydrurga leptonyx*), and crabeater (*Lobodon carcinophagus*) seals which give birth and breed in the pack ice surrounding Antarctica, were not geographically structured. However, breeding colonies of Weddell seals (*Leptonychotes weddellii*) sampled in the in the land-fast ice of Antarctica were moderately structured. In contrast, the Arctic pack ice breeding bearded seal (*Erignathus barbatus*) was strongly structured

throughout most of its circumpolar distribution, while populations of the land-fast ice breeding ringed seal (*Phoca hispida*) were genetically homogenous.

The contrasting pattern of population structure, in relation to breeding habitat, among Antarctic and Arctic ice breeding seals was examined in a phylogenetic context. The evolution of pagophilic behaviour in the Antarctic ice breeding seals has led to the loss of population structure in most species. However in the Arctic, the process leading to the evolution of population structure has been influenced by a cold adapted, ice breeding ancestor that was unable to move through areas of thick ice.

Acknowledgements

I am grateful to my supervisor, Curtis Strobeck, for providing the intellectual and financial support that made this project possible. The success of the research presented in this thesis is due, in no small part, to the contributions of Ian Stirling. He openly shared both his knowledge and his personal contacts that were requisite in the pursuit of the research presented here. Without these things and his "sharp stick" the pages that follow would never have come to be.

Both the scope and success of my project depended on the collection of many samples in inhospitable places. I am indebted to all the individuals and agencies that collected the samples used here. Many thanks in particular to W. Amos, S. Atkinson, J. Beauchesne, J. Bengston, M. Bester, A. Blix, P. Boveng, I. Boyd, H. Burton, M. Campbell, M. Cameron, A. Collet, R. Dietz, N. Gemmell, Grise Fiord Hunters and Trappers Organization, F. Gulland, L. Harwood, S. Iverson, K. Kovacs, L. Lowry, C. Lydersen, Y. Naito, E. Nordoy, T. Oritsland, J. Plotz, B. Robson, T. Rogers, K. Sato, P. Shaughnessy, B. Sjare, C. Sonne-Hansen, C. Southwell, and M. Wainstein. You made this project possible.

I thank Don Siniff for the opportunity to expand this project to the scope presented here and also for the chance to visit Antarctica. I thank Tom Gelatt for his patient and giving nature and for encouraging my brief but unforgettable escape from the lab to go and meet the seals. To the "gene team" and the "blood suckers" (B. Stewart, P. Yochem, M. Koski, M. Castellini, T. Mau, and S. Trumble) thanks for the good times both on and off the ice.

I am thankful to the members of the Strobeck lab, Canadian Wildlife Service, and the Department of Biological Sciences who have contributed to the work here in one way or another: D. Andriashek, J. Brzustowski, W. Bernhardt, W. Calvert, L. Carmichael, V. Campbell, J. Coffin, D. Coltman, I. Delisle, G. Dueck, M. Holst, J. Jelielek, N. Keyghobadi, C. Kyle, C. Lausen, M. McPherson, D. Paetkau, R. Polziehn, C. Popplewell, M. Proctor, H. Rai, E. Richardson, G. Wilson, M. Vonhof, and K. Zittlau.

This project was generously funded by NSERC, and NSF. NSF and the Department of Biological Sciences provided personal funding during my graduate studies.

Without the support of my family and Jennifer Bonneville I would not have had the strength to pursue and complete this thesis. To them I am indebted.

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Chapter 1
General Introduction

Overview of the Pinnipedia

The Pinnipedia have long been recognized as a unique group of marine carnivores. The group consists of three families and 33 living species distributed worldwide (Rice, 1998). The three families are the Odobenidae (walruses; one species), the Otariidae (fur seals and sea lions; 14 species) and the Phocidae (true or earless seals; 18 species). There has been extensive debate over the evolutionary relationships between pinniped families, and their origins among the carnivores (reviewed in Berta, 2002). After roughly a century long debate, there is now general acceptance of the monophyly of the Pinnipedia, and the recognition that they represent a distinct lineage within the caniform (dog-like) carnivores. However, questions relating to relationships within the Phocidae (Bininda-Emonds and Russell, 1996) and Otariidae (Wynen *et al.*, 2001) still persist. Irrespective of these persisting phylogenetic problems, it is known that the pinnipeds arose from a terrestrial carnivore stock of either ursid (bear) or mustelid (weasel) origin. A consequence of this evolutionary history is that the distribution of all pinniped species is essentially coastal or associated with ice in polar regions. Although pinnipeds forage at sea and can maintain a pelagic existence for long periods of time, they must leave the water for parturition, maintaining their ties to a terrestrial (or ice) habitat. Therefore, the two most important features of pinniped life history are marine feeding and terrestrial parturition (Bartholomew, 1970)

Pinniped mating systems

In general, fur seals and sea lions (Otariidae) are confined to the Pacific Ocean basin, have temperate distributions, and breed on land. In contrast, the true seals (Phocidae) are found in both the Pacific and Atlantic basins. Of the eighteen species of true seals, ten are found in polar regions and breed in the water below land-fast (Weddell and ringed, seals) and pack ice (crabeater, leopard, Ross, bearded, hooded, harp, ribbon, and spotted

seals). Three are distributed in temperate regions, at higher latitudes, and mate on (southern elephant seal) or in the water near land (grey, and harbour seals), although they may also breed on, or in the water near, ice in certain locations. Three species have an equatorial distribution and breed on land (northern elephant seal), or in the water (Mediterranean and Hawaiian monk seals) adjacent to it, while two species are land locked endemics (Caspian and Baikal seals). Our understanding of the distribution of most pinniped species is based primarily on the location of breeding colonies and less is known about their pelagic distribution.

Extensive consideration has been given to the evolution of mating systems in pinnipeds. Most authors agree that access to, and stability of, habitat for parturition has been the most dominant factor influencing the evolution of mating systems in pinnipeds (Bartholomew, 1970), although other aspects of life history, and phylogenetic constraints have also contributed. This is because birth of pups must occur out of the water, females develop fidelity to suitable pupping sites, mating occurs shortly after parturition and is highly synchronized in time, and males are attracted to the presence of estrous females (Stirling, 1983).

In general, pinnipeds use three types of habitat for parturition. Habitats for pupping are terrestrial (islands and ice free coastal areas), pack ice (free floating), or land-fast ice (contiguous with islands or continents). For species that pup on land, females develop fidelity to locations that are predictable. Space for parturition in these locations is often limited and females must give birth at high densities. Under these conditions males can compete for residence in areas where females congregate and as a likely consequence extensive polygyny, and sexual dimorphism have resulted. Species that mate on land likely have higher levels of polygyny than those that mate aquatically due to the increased ability for males to exclude competitors in a relatively limited two dimensional (land), rather than a less limited three dimensional (water) environment. In contrast, pack ice is an unlimited and unstable habitat for parturition. The females of species that use floating pack ice for parturition are widely spaced and males can not restrict assess to more than one, or possibly a few, estrous females, and polygyny is consequently unlikely to develop. As the substrate for pupping is relatively unlimited and distributed uniformly, fidelity for breeding areas is not expected. The effect of use of

land-fast ice for pupping and mating on the development of polygyny is expected to be intermediate to that of pack ice and terrestrial habitat. Although land-fast ice is a potentially unlimited substrate for parturition, pupping females only use areas where predictable breathing holes form. Because the number of breathing holes is limited, many females access the water through the same holes. Males can defend territories in the water below breathing holes and may be able to mate with multiple females. Polygyny is expected, but not to the degree seen in land breeding species, because males must defend three-dimensional territories. Breathing holes form in predictable areas of the land-fast ice each year, and so females are likely to develop fidelity to breeding sites.

Molecular studies of mating systems

Assessment of mating structure in phocids and walruses is difficult because, for some species, mating occurs in the water and cannot be directly observed. Molecular techniques have been used to assess male mating success in a number of species that breed colonially including grey seals (Worthington Wilmer *et al.*, 1999), harbour seals (Coltman *et al.*, 1998), northern and southern elephant seals (Hoelzel *et al.*, 1999), Weddell seals (Gelatt, 2001) and Antarctic fur seals (Gemmell *et al.*, 2001). Among the phocid species examined, terrestrially breeding northern and southern elephant seals displayed higher levels of polygyny than aquatically mating grey, harbour, and Weddell seals. In the single otariid species examined (Antarctic fur seals; Gemmell *et al.*, 2001), polygyny was much lower than expected, given that breeding is terrestrial and females are present in high densities in colonies on land. Molecular studies examining male mating success have not been performed in any pack ice breeding species, due to the difficulties of sampling likely fathers, and their pups the following season. Empirical examinations of mating systems in pinniped species generally support theories about mating structure in relation to breeding habitat but the lack of studies on pack ice breeding species illustrates the need for additional studies.

Assessment of population structure

Differences in breeding habitat preference may be related to the evolution of mating systems in pinnipeds and have also likely contributed to the patterns of population structure in most species. For species that use terrestrial breeding areas, the predictability and stability of this habitat may cause individuals to show high levels of site fidelity. In contrast, the unlimited and uniform characteristics of the pack ice breeding habitat may lead to large inter-annual movements of individuals throughout their widely dispersed breeding range. Cracks used as breathing holes during breeding in the land-fast ice habitat of polar regions form in predictable locations but annual variations in ice conditions make this habitat less predictable than terrestrial pupping sites. Therefore species that use land-fast ice for parturition and mating may show levels of site fidelity intermediate to pack ice and terrestrial breeding species.

Traditionally, the assessment of population subdivision in pinnipeds has been performed using mark recapture and telemetric methods. However, molecular genetic methods may offer a more direct determination of population structure, especially for species where direct observation of a large number of individuals is difficult due to the relative inaccessibility of their habitat. Hoelzel (1997) reviewed the current status of pinniped population genetic studies and indicated that, due to the limited number of population genetic studies that had been performed on pinniped species, major patterns of population differentiation across species could not be determined (Hoelzel, 1997).

Project summary

In order to gain a more comprehensive understanding of the effects of breeding habitat on the development of population structure in phocid species, a major aim of this study was to investigate the population genetic structure of four species of antarctic (crabeater, leopard, Ross, and Weddell) and two species of arctic (bearded and ringed) ice-breeding seals. The results of these studies were used, in conjunction with previously published studies examining population differentiation in other phocid species, to compare trends in population structure in relation to breeding habitat selection, ecology, phylogeny, and life

history characteristics of each species (Chapter 5). Phylogenetic relationships have likely played a role in the evolution of mating systems and the development of population structure in phocid species. Therefore, I used complete mitochondrial coding sequences to investigate the phylogenetic relationships among members of the Phocidae (Chapter 2). Microsatellite markers were previously available for northern phocid species but were of limited use in antarctic species (Gemmell, 1997). Therefore, I developed microsatellite markers for use in the antarctic seal species. Details of microsatellite isolation and characterization in other species are given in Chapter 6. These microsatellite markers were used to assess population structure in four species of antarctic ice breeding seals and the results of this analysis are given in Chapter 3. A subset of the markers used in the antarctic species were used in conjunction with previously developed markers from grey seal (Allen *et al*, 1995) and harbour seal (Gemmell et al, 1997) to assess population structure in bearded and ringed seals (Chapter 4).

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Chapter 2

A phylogeny of the extant Phocidae inferred from complete mitochondrial DNA coding regions

Introduction

The true or earless seals (Class Mammalia: Order Carnivora: Family Phocidae) have long been recognized as a unique group of marine mammals. Together with the Otariidae (sea lions and fur seals) and the Odobenidae (walrus), they constitute the extant members of a higher level group of aquatic carnivores termed Pinnipedia. Historically, phylogenetic investigations involving the Phocidae have focused on their placement within the Pinnipedia and in turn on the relationship of the three pinniped families to the terrestrial carnivores. Although the arctoid (bear-like) affinities of all pinnipeds are readily accepted, there has been significant debate over whether the pinnipeds originated from one (monophyletic) or two (diphyletic) ancestral stock(s) (Mitchell and Tedford, 1973). Related to this debate is the question of which specific group of terrestrial carnivores gave rise to the pinnipeds (Flynn *et al.*, 1988). Evidence from early morphological, biogeographical, and paleontological studies has often supported a diphyletic origin of the pinnipeds (Tedford, 1976; Ray, 1976; de Muizon, 1982), whereby the Otariidae and Odobenidae are attributed a common ursid (bear) ancestry while the phocids are granted mustelid (weasel) affinities. These ideas deviate from the more recent conclusions of molecular and karyological studies that propose a monophyletic Pinnipedia of ursid ancestry where an odobenid - otariid clade is the sister group of the phocids (Sarich 1969; Árnason 1977; de Jong 1982; Vrana *et al.*, 1994; Árnason *et al.*, 1995; Lento *et al.* 1995). In contrast to earlier morphological studies, more recent ones now increasingly agree with the notion of a monophyletic Pinnipedia, however these studies suggest a common odobenid - phocid clade that is the sister group of the otariids (Wyss, 1987). This higher-level organization has gained much support and is now the prevailing view of pinniped phylogeny (Flynn 1988; Vrana *et al.*, 1994).

The extensive attention to family level relationships and to ancestral affinities of the pinnipeds is in marked contrast to the amount of study that has been focused on

relationships below the family level. The monophyly of the Phocidae has not been challenged since the group was originally granted familial status by Gray in 1821, and the species composition of the group has stayed relatively constant over this time. Early taxonomies of the Phocidae included four subfamilies: the Cystophorinae (hooded and elephant seals), Lobodontinae (antarctic seals), Monachinae (monk seals), and the Phocinae (other northern hemisphere seals). The distinction between the Lobodontinae and the Monachinae was deemed to be primarily one of geography and as such the two subfamilies were demoted to tribes (Lobodontini and Monachini respectively) within a newly defined Monachinae (Scheffer, 1958). The Cystophorinae was dismantled owing to the recognition that the morphological characters used to define the group are likely convergent, and as such the hooded seal and elephant seals were placed into the tribes Cystophorini and Miroungini, within the Phocinae and Monachinae respectively (King, 1966). Therefore two subfamilies within the Phocidae are recognized today: the Monachinae or "southern" hemisphere seals (including the antarctic, elephant and monk seals) and the Phocinae or "northern" hemisphere seals (including the bearded, hooded, harp, ribbon, grey, ringed, harbor and spotted seals; see Table 2-1).

Recently, the monophyly of the Monachinae has been questioned (Repenning and Ray, 1972; Wyss, 1988), in large part due to the uncertain relationship of the monk seal species (*Monachus* spp.) to each other and to the remaining phocids (de Muizon , 1982). The primitive nature of the monk seals (de Muizon, 1982; King, 1983) has led to the longstanding idea that the group is monophyletic, however recent arguments have suggested that the characters used to define *Monachus* are symplesiomorphic, and that in fact the group may be paraphyletic and ancestral to the remaining phocids (Wyss, 1988). Additionally, the recent morphological study of Bininda-Emonds and Russell (1996) suggested that undue emphasis on the primitive features of *Monachus*, at the expense of more derived ones, has led to the traditional belief that *Monachus* is ancestral, and instead placed a monophyletic *Monachus* within the Lobodontini (antarctic seals) thus rendering the subfamily paraphyletic. The only molecular study to include members of the Monachinae (Árnason *et al.*, 1995) supported a basal position of *Monachus* relative to the remaining phocids and monachine paraphyly, although support for this arrangement was weak. As only one species of monk seal was sampled in that study the monophyly of

Monachus could not be addressed. Relationships within the Lobodontini are poorly understood. A single study of cranial morphology supported grouping Ross and Weddell seals as the sister group of a crabeater – leopard seal clade (Hendey, 1972). Although the monophyly of *Mirounga* spp. (elephant seals) has never been questioned, both extant species have not been included in a molecular phylogenetic study and therefore their monophyly has not been tested. The utility of tribal distinctions within the Monachinae has been questioned (Henday and Repenning, 1972), due to the fluctuating species composition of the group over time, combined with a suggestion of possible paraphyly of the group. Although current taxonomy includes all "southern" hemisphere seals in the Monachinae, the validity of the three recognized tribes, Lobodontini (antarctic Seals), Miroungini (elephant seals), and Monachini (monk seals), requires reexamination.

Monophyly of the Phocinae has not been questioned since King (1966), yet considerable debate exists over relationships within the group. Much of the confusion may be caused by the hypothesized recent rapid radiation among members of the tribe Phocini (Ray; 1976; Árnason *et al.*, 1995). The general lack of resolution within this group has led to questions regarding generic distinctions. The ringed seal (*Pusa hispida*), harp seal (*Pagophilus groenlandica*), and ribbon seal (*Histriophoca fasciata*) have alternatively been given their own generic status or have been included as subgenera within the genus *Phoca*. Although the grey seal (*Halichoerus grypus*) has long been recognized as distinct, the merit of its generic status has been questioned and may be unjustified if the same rank is not granted to the other putative subgenera of *Phoca* (Árnason *et al.* 1995; Perry *et al.* 1995). The recognition that *Halichoerus grypus* is more closely related to *Phoca vitulina* (harbor seal) and *Pusa hispida* (ringed seal) than either of these are to *Pagophilus* and *Histriophoca* has been the justification for generic status of these later taxa. In the taxonomy presented in Table 2-1, *Phoca* is used in its strictest sense, including *Phoca vitulina* (harbor seal), and *Phoca largha* (spotted or largha seal). The generic names *Pusa*, *Pagophilus*, and *Histriophoca* are also preferred. An alliance of *Cystophora*, the only genus of the tribe Cystophorini, with *Pagophilus* and *Histriophoca* has been proposed (de Muizon, 1982; Perry *et al.*, 1995) and would render the Phocini paraphyletic. The long accepted basal status of *Erignathus*, the monotypic genus of the tribe Erignathini, has recently been questioned (Bininda-Emonds and Russell, 1996) for

reasons similar to those proposed for *Monachus* above. Their recognition that *Erignathus* possesses apomorphies of the Phocini clade suggests the inclusion of *Erignathus* within the tribe Phocini making phocine tribal appellations inappropriate.

The current problems in phocid systematics outlined above (see Figure 2-1) may be due to a general lack of study within the group at the species level. Cladistic studies of seals utilizing morphology are rare, and often suffer from the restriction of limited sampling above the species level, therefore assuming the monophyly of higher-level taxa, not allowing for novel species pairings that could result from a species level analysis. While molecular studies have been performed at the species level, they have mainly only included members of subfamily Phocinae (Perry *et al*, 1995; Mouchaty *et al.*, 1995). The only study to include complete species sampling and a cladistic analysis (Bininda-Emonds and Russell, 1996) proposed many novel relationships within the Phocidae that may require careful review. Taken as a whole, species relationships within the Phocidae are poorly understood and require reexamination using a rigorous methodology, a large dataset, and complete taxa sampling.

Recent advances in the field of molecular biology have allowed for the collection of large molecular data sets, often comprising the complete genomes of organelles (Árnason *et al.*, 1993, 2002; Lin *et al.*, 2002; Delisle and Strobeck, 2002). The use of large, multi-gene data sets in phylogenetic construction not only provides the numerous informative changes required for inference of correct topologies and may also help to raise a weak phylogenetic signal above the noise level (Bull *et al.* 1993). The development of new tree building methods (Huelsenbeck *et al.*, 2001) and tests for assessing the statistical support of specific topologies (Swofford *et al.*, 1996; Shimodaira and Hasegawa, 1999; Goldman *et al.*, 2000) have improved phylogenetic inference and our ability to assess confidence in the obtained trees. Also, the availability of tissue samples from rarely observed species permits a more complete taxon sampling than was available in previous molecular studies. These recent developments in molecular biology, phylogenetic inference, and tissue availability allows for a robust assessment of the systematic relationships within the Phocidae, their relationship to the remaining pinnipeds, and their sister group relationships.

To address these relationships, and the specific issues described above, we used the complete coding sequence of the twelve heavy-strand encoded genes of the mitochondrial DNA molecule. All extant members of the Phocidae were included in the analysis except the two landlocked endemic freshwater species (*Phoca siberica* and *Phoca caspica*) and the ribbon seal (*Histriophoca fasciata*). Four otariids, including two fur seals and two sea lions, and the only living member of *Odobenus*, the walrus, were included as outgroups to the phocids. In order to assess the affinities of the true seals, and possible diphyly of the Pinnipedia, we included three ursid and two mustelid species. The inclusion of members of both putative relatives allows the analysis to dictate the most closely related outgroup taxon without *a priori* assuming that a particular outgroup is the sister group. The inclusion of one canid and one felid rooted the tree within the Carnivora. Phylogenetic construction was performed using maximum parsimony (Farris, 1970; Fitch, 1971), maximum likelihood (Felsenstein, 1981), and Bayesian approaches (Rannala and Yang, 1996; Larget and Simon, 1999). Regions of the inferred trees that showed weak support by traditional methods, or differences using alternative tree building methodologies, were tested against other potential topologies using recently developed statistical tests (see Goldman *et al.*, 2000). Data from three additional mitochondrial regions were used in conjunction with the sampling of geographically distant conspecifics in order to assess the ability of additional data to resolve poorly supported regions of the inferred phylogeny. Results of the analysis were used to assess the accuracy of current taxonomy of the phocids.

Materials and Methods

Samples, Sequencing Strategy, and Molecular Methods

Tissue samples (skin, muscle, or blood) were obtained from individuals of the following species: *Lobodon carcinophagus* (crabeater seal), *Ommatophoca rossii* (Ross Seal), *Hydrurga leptonyx* (leopard seal), *Leptonychotes weddellii* (Weddell Seal), *Mirounga angustirostris* (northern elephant seal), *Mirounga leonina* (southern elephant seal), *Erignathus barbatus* (bearded seal), *Cystophora cristata* (hooded seal), *Pagophilus*

groenlandica (harp seal), *Pusa hispida* (ringed seal), *Phoca largha* (spotted seal), *Otaria byronia* (South American seal lion), *Arctocephalus australis* (southern fur seal), *Odobenus rosmarus* (walrus), *Gulo gulo* (wolverine), and *Mustela vison* (American mink). I employed a PCR based strategy (Delisle and Strobeck, 2002) to amplify and sequence an approximately 13000 bp region of the mitochondrial DNA molecule containing genes encoding ATP synthase subunits 6 and 8, cytochrome oxidase subunits I, II and III, and NADH dehydrogenase subunits 1, 2, 3, 4, 4L, and 5. The region containing the cytochrome b gene was sequenced for species where no published sequence was available in GenBank (*A. australis*, *O. byronia, M. monachus, O. rossii, L. carcinophagus, M. angustirostris*), and for *M. leonina*, *L. weddellii*, and *H. leptonxy*. The remaining sequence of the complete mitochondrial DNA molecule containing the light-strand encoded NADH dehydrogenase subunit 6, the two ribosomal RNA genes, 12S rRNA and 16S rRNA, was sequenced for the two elephant seal species (*M. angustirostris*, and *M. leonina*) and the four antarctic species (*L. carcinophagus*, *O. rossii*, *H. leptonyx*, and *L. weddellii*). An approximately 6700 bp region containing 7 coding genes was sequenced for a second individual of *M. angustirostris*, *L. carcinophagus*, *O. rossii*, *H. leptonyx*, and *L. weddellii* sampled from geographically distant locations relative to the other sequenced individual.

Total genomic DNA was extracted from tissue samples using DNeasy Tissue Extraction Kit (Qiagen), and approximately 250 ηg was used as template in 100 μL reactions also containing 0.4 μM of each primer, 160 μM dNTP's, 2.5 mM $MgCl_2$, 3 units of Taq DNA polymerase (isolated as in Engelke *et al.* 1990) and 1X PCR buffer (10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 50 mM KCl, and 0.16 mg/mL bovine serum albumin). PCR reactions were performed in a Perkin Elmer 9600 thermal cycler under the profile: 3 min at 94°C followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 90 seconds). Resulting fragments were resolved in 0.8% agarose gels and sequencing template was isolated from excised bands using the QIAquick gel extraction kit (Qiagen). Sequencing was performed using Applied Biosystems dRhodamine or BigDye (v.3.0) sequencing chemistries, under the manufacturer recommended protocols, and resolved on an ABI 377 automated DNA sequencer. Primers for amplification and sequencing are from Delisle and Strobeck (2002). Amplification of fragment mtDNA10

(Delisle and Strobeck, 2002) using the original primers was problematic, likely due to the length of the product, therefore new primers were designed to facilitate the amplification of this region in two fragments (here named mtDNA fragment 10A and 10B). Other additional primers were designed when existing primers failed to amplify successfully or resulted in poor quality sequences. Primer sequences for fragment amplification and sequencing of each species are given in Table 2-6.

DNA sequences for the mitochondrial heavy-strand coded genes of 12 additional phocid and outgroup species were extracted from previously published complete mitochondrial genomes including: *Felis catus* (domestic cat; NC_001700; Lopez *et al.*, 1996), *Canis familiaris* (domestic dog; NC_002008; Kim *et al.*, 1998), *Ursus americanus* (American black bear; NC_003426), *Ursus arctos* (brown bear; NC_003427), *Ursus maritimus* (polar bear; NC_003428);(Delisle and Strobeck, 2002), *Phoca vitulina* (harbor seal; NC_001325; Árnason and Johnsson, 1992), *Halichoerus grypus* (grey seal; NC_001602; Árnason *et al.*, 1993), *Eumetopias jubatus* (Stellar sea lion; NC_004030; Árnason *et al.*, 2002), and *Arctocephalus forsteri* (New Zealand fur seal; NC_004023; Lin *et al.*, 2002). In addition published cytochrome b sequences were obtained from: *M. schauinslandi* (X72209; Árnason *et al.*, 1993), *E. barbatus* (X82295), *C. cristata* (X82294), *P. groenlandica* (X82303), *P. hispida* (X82304), *P. largha* (X82305), *O. rosmarus* (X82299)(Árnason et al., 1995), *G. gulo* (AB051245; Hosoda *et al.*, 2000) and, *M. vison* (AB026109; Kurose *et al.*, 2000).

Phylogenetic analysis

Sequences were analyzed, basecalled and assembled using Sequence Analysis v3.4.1, and Sequence Navigator v1.0.1 (Applied Biosystems). Novel sequences and those obtained from Genbank were used to create a NEXUS matrix containing the sequence of 12 mitochondrial genes for 27 species. Where length variation existed between species, sequences were aligned with respect to inferred amino acid sequence. Sequence data were partitioned by gene (12 partitions) and codon position (3 partitions). Congruence among data sets was assessed by examining bootstrapped maximum parsimony trees (100 repetitions, 10 addition sequence replicates) of each gene and coding position for the

existence of "hard" incongruencies. In addition, the incongruence length difference test (Farris *et al.*, 1995) as implemented in PAUP* (version 4.0b10; Swofford, 2000) was used to assess congruence among data partitions. Tests were performed over all gene partitions (one test), from each gene to the remaining genes (12 tests), and pair-wise between each gene (66 tests), over all codon positions (one test), from each codon position to the remaining positions (3 tests) and pair-wise between codon positions (3 tests).

Heuristic tree searches using maximum parsimony in PAUP* (version 4.0b10; Swofford, 2000) were performed using 10 random stepwise addition replicates and TBR branch swapping. Bootstrap support values for the inferred tree were assessed by non-parametric data re-sampling (1000 replicates, 10 random stepwise addition replicates per bootstrap replicate). Maximum likelihood tree searches were performed using a GTR + I + Γ model of sequence evolution (Rodríguez *et al.*, 1990) with four discrete Γ distribution rate classes. This model of sequence evolution was chosen using Modeltest version 3.06 (Posada and Crandall, 1998). Initial model parameters were estimated on the maximum parsimony tree and were refined together with tree topology over two rounds of successive parameter estimation and heuristic searching using TBR branch swapping. A minimum evolution distance tree was constructed using the neighbor joining algorithm to determine a starting tree and with estimates of pair-wise distances derived using the GRT + I + Γ substitution parameters from the ML analysis. Pair-wise sequence differences were plotted against taxonomic rank for three naming schemes to assess the correlation of current taxonomy with genetic divergence. MrBayes (version 3.0B4; Huelsenbeck and Ronquist, 2001) was used as a comparative tree searching method to those implemented in PAUP. As above a GTR + I + Γ model of evolution was used. A total of 1 million generations were run using a random starting tree and four chains (three heated using the default value of 0.2). Trees were sampled every 100 generations, and the first 5000 trees were discarded as burnin. A majority rule consensus tree of the last 5000 sampled trees was used to assess the Bayesian posterior probability (BPP) support for clades found in the consensus tree.

Statistical tests of alternative topologies

Two recently developed statistical tests (see Goldman *et al.*, 2000) were used to evaluate alternative topologies around nodes found to have weak support using the tree building and bootstrapping methodologies described above. Three nodes in the tree had poor support, each representing a four-taxon problem. Therefore, relative support for each of the three topologies was estimated using the Shimodaira-Hasegawa test (SH test; Shimodaira and Hasegawa, 1999) based on the complete data set. Due to the intense computational requirements of the SOWH test (Swofford *et al.*, 1996) representative subsets of the complete data matrix were selected to facilitate pair-wise tests between competing topologies. For comparison to the complete data set, SH tests were also run on the restricted taxa sets.

Thirteen taxa representing the major lineages found in the MP, ML and Bayesian analysis were selected for an examination of alternate hypotheses regarding pinniped ancestral affinities. Two competing topologies were tested, in a pair-wise manner, to the ML tree, using a GTR + I + Γ model of sequence evolution as selected using the likelihood ratio test implemented in Modeltest v3.06 (Posada and Crandall, 1998). Model parameter estimates, and likelihood scores, were calculated for each topology in PAUP and parameters for alternative topologies were used to generate 100 parametric bootstrap data sets in Seq-Gen (v1.2.5; Rambaut and Grassly, 1997). A partial optimization of the SOWH test (the second of two partial optimization "posPpud" tests in Goldman *et al.*, 2000) was used to test support for the alternative topology in two independent SOWH tests.

Relationships among the Phocini were tested using the seven sampled species of "northern" phocids (Phocinae). Here, due to the selection of a less sophisticated sequence evolution model in Modeltest (version 3.06; Posada and Crandall, 1998), and the use of fewer taxa in the analysis, the full optimization of the SOWH test was implemented (posPfud in Goldman *et al.*, 2000). The HKY + Γ (Hasegawa *et al.* 1985) model of sequence evolution (as selected in Modeltest version 3.06) was used to generate 100 replicate data sets in Seq-Gen using the model parameters and topology of the

alternative hypotheses. As above, two SOWH tests were performed comparing the likelihood score difference between the two alternative topologies and the ML score.

A statistical examination of relationships among the Lobodontini followed as above, using the eight sampled "southern" hemisphere seals except with a TN93 + I + Γ (Tamura and Nei, 1993) model of sequence evolution. Again, two tests were performed using the full optimization of the SOWH test.

The ability of additional data to resolve the question of monachine phylogeny was examined. Here a taxon set restricted to an even greater degree, such that it included only the four antarctic species and the two elephant seals, was used. In order to address the effect of the addition of an increased number of phylogenetically informative characters, three additional mitochondrial sequences (ND6, 12S rRNA, and 16S rRNA) were added for these six species. Likewise, the effect of increased taxon sampling was examined by utilizing the sequence of a second, geographically distant conspecific individual for five of these six species. Here results of the SH and SOWH tests were compared with results using these six taxa and the original twelve gene matrix. Testing was performed as above using the TN93 + Γ model of sequence evolution as selected using Modeltest.

Results

The sequences of the twelve heavy-strand coded genes of the mitochondrial genome were successfully determined for all taxa, and have been deposited in Genbank (Table 2-2). The order of genes was identical, in all species, to previously sequenced phocid and other mammalian mitochondrial genomes. The resulting data matrix included a total of 10842 aligned characters providing, 5210 variable sites, of which 4390 were parsimony informative. Length variation was observed in only one gene, NADH dehydrogenase subunit 5 (ND5), near the 3' end of the sequence. ND5 was 1836 bp long in the otariid species and the American mink (*M. vison*) but was 6 bp shorter (1830 bp) in the remaining species except the Mediterranean monk seal (*M. monachus*) where it was 1827 bp. The length of all other genes was constant across species allowing for unambiguous alignment of characters in the data matrix.

Examination of maximum parsimony trees and their bootstrap support for each gene revealed no "hard" incongruencies. The topology of trees derived from each gene differed from each other and from the tree found using the complete concatenated sequence. However, these differences occurred in areas of weak support or around short branches. The ILD test (Farris *et al.*, 1995) indicated no significant heterogeneity among data set partitions. Comparisons of all genes to each other, and each gene to the remaining data all revealed non-significant results. Two of 66 pair-wise tests between individual genes yielded significant results (ATP6:ND5; p=0.04, and ND3:ND5; p=0.02). ND5 was not excluded from the analysis, even though it was implicated in both significantly incongruent single gene comparisons, as the analysis of heterogeneity between this data partition and the remaining genes was not significant (p=0.07). No significant heterogeneity was observed in comparisons of all codon positions to each other, each position to the remaining data, or in pair-wise comparisons. Therefore the complete data set was used to infer phylogenetic relationships using MP, ML, and Bayesian methods.

The maximum parsimony search revealed a single most parsimonious tree (TL = 19760; CI = 0.390, RI = 0.536). The MP tree was identical to the tree found using maximum likelihood (-lnL = 91756.35) and the Bayesian analysis, except that the former supported a sister group relationship of the Mustelidae and Pinnipedia (MP bootstrap = 58%, Bayesian posterior support 1%), while the latter two methods supported an ursid - pinniped clade with a mustelid sister group (Figure 2-2). Bayesian posterior probability support (BPP) was highest (97%) for the ursid sister group arrangement supported by ML; support for this arrangement under MP was only 18%. The placement of Ursidae and Mustelidae as a clade, sister to the Pinnipedia was weakly supported (MP bootstrap 21%, BPS 2%). All analyses supported a well-defined split of the Arctoidea from the remaining Caniformia (MP bootstrap 93%, BPS 100%).

Ingroup relationships within the Pinnipedia were identical, irrespective of the tree searching methodology used (Figure 2-2). Within a strongly supported, monophyletic Pinnipedia, the three families Phocidae, Otariidae, and Odobenidae were also found to be monophyletic. The presence of a common otariid - odobenid clade (MP bootstrap 100%, BPS 100%) as sister to the Phocidae was also robustly supported.

Within the Phocidae, monophyly of both the Phocinae and Monachinae (including the monk seals) was supported with 100% MP bootstrap and BPS support (Figure 2-2). *Erignathus* was found to define the most basal split within the Phocinae, being the sister group of the remaining "northern" hemisphere seals. Among the remaining members of the Phocinae, *Cystophora* was placed as the sister group of the Phocini. Within this tribe, *Pagophilus* was the sister group of the *Phoca* species. Except for the strongly supported grouping of *P. vitulina* and *P largha*, relationships among the remaining *Phoca* species and *Halichoerus* were poorly resolved, with marginally more support favoring a sister relationship of *H. grypus* and *P. hispida*. The bulk of the remaining bootstrap support favored a position of *P. hispida* as the sister groups of the other tree taxa while almost no support was found for a deep branching position of *Halichoerus* (Figure 2-2b).

Strong support for three lineages was found within the Monachinae. The two *Monachus* spp. included here form a monophyletic group with strong bootstrap (99%) and Bayesian posterior clade support (100%). Both *M. schauinslandi*, and *M. monachus* appeared as ancient lineages originating only shortly after the diversification of the monachines, and are the sister group of the remaining "southern" hemisphere seals. The elephant seals (*Mirounga* spp.) form a monophyletic clade, sister to the lobodontines. Within the lobodontines, the Weddell and leopard seals were grouped together to the exclusion of the crabeater and Ross seals, however relationships of the latter two species were contentious. Maximum parsimony, maximum likelihood, and Bayesian analysis all favored the placement of *O. rossii* as deepest branching within the antarctic seals, however considerable support was also found for a basal position of *L. carcinophagus*, while there was very little support for a sister relationship between *Lobodon* and *Ommatophoca* (Figure 2-2a).

Using the complete dataset, the SH test (Shimodaira and Hasegawa, 1999) was unable to discriminate between the three alternative topologies concerning the ancestral affinities of the Pinnipedia (Table 2-3a). The ML tree was the least parsimonious of the three competing trees (TL 19770) requiring 10 additional steps relative to the MP topology. Differences in likelihood, as compared to the ML tree, were slight (4.00332 and 4.44803) resulting in an inability to reject ($p = 0.467$; [(mustelid, pinniped), ursid] and $p = 0.430$; [pinniped (mustelid, ursid)]) the alternative topologies. Likewise, the SH

test could not distinguish between topologies regarding the relationship of *P. hispida* and *H. grypus* within the Phocini (Table 2-3b). Although, the arrangement of these two species on a common branch, sister to *P. vitulina* and *P. largha* was supported by both MP and ML, the alternative topologies were not statistically rejected [p = 0.627 (hispida(grypus(vitulina,largha))); and p = 0.186 (grypus(hispida(vitulina,largha)))]. Among the lobodontines, the basal position of *O. rossii* was supported by both MP and ML, however, as before, alternative topologies could not be rejected using the SH test (Table 2-3c).

Using a subset of the twenty-seven sampled taxa (*L. weddellii, M. schauinslandi, P. hispida, E. barbatus, A. australis, O. byronia, O. rosmarus, U. arctos, U americanus, G. gulo, M. vison, C. familaris, and F, catus*), two equally parsimonious trees were recovered (Table 2-4a). One of these trees, placing the Ursidae as the sister group to the Pinnipedia, was also recovered using ML. Using the SH test (Shimodaira and Hasegawa, 1999) the alternative topologies of a sister group relationship of the Mustelidae to the Pinnipedia, and a common ursid-mustelid branch sister to the pinnipeds, could not be rejected as equally good explanations of the data. However, the partial optimization of the SOWH test ("posPpud" in Goldman *et al.*, 2000) suggested that the alternative topologies should be excluded as the correct topology.

Using only the members of the Phocinae, (*E. barbatus, C. cristata, P. groenlandica, P. hispida, H. grypus, P. vitulina, and P. largha*) the SH test was again unable to reject any alternative topologies (Table 2-4b). However the full optimization of the SOWH test (posPfud in Goldman *et al.* 2000) weakly (p=0.02) supported the rejection of a basal position of *H. grypus*, in favor of either an ancestral position of *P. hispida*, or a *H. grypus* - *P. hispida* clade as sister to the remaining *Phoca* species.

A similar analysis was performed, examining the placement of the crabeater seal (*L. carcinophagus*) and the Ross seal (*O. rossii*) among the lobodontines using the eight sampled members of the Monachinae. Again, the SH test was unable to discriminate among the three alternative topologies (Table 2-4c). The results of the full optimization of the SOWH test suggested the tree placing *L. carcinophagus* in a basal position could be weakly rejected (p=0.05), however the tree containing an *Ommatophoca* - *Lobodon* clade as sister to the remaining lobodontines could not be rejected (p=0.09).

The utility of additional sequence data to help resolve relationships within the Lobodontini was limited. Using only six species (*M. leonina, M. angustirostris, L. carcinophagus, O. rossii, L. weddellii, and H. leptonyx*), and either the original 12 gene data set or this data set in conjunction with the data from three additional mitochondrial DNA regions, ML consistently recovered trees with *L. carcinophagus* as the deepest branching species within the lobodontines (Table 2-5a and 2-5b). This is in contrast to the use of the complete taxon set, where *O. rossii* was placed as the deepest taxon (see Figure 2-2). The SH test was unable to reject any of the three alternate topologies using either the 12 gene or 15 gene data set. Using only the 12 gene data set the SOWH test did not reject either a basal position of *O. rossii*, or a common *Ommatophoca - Lobodon* clade. However, with the 15 gene data set, the SOWH test suggested rejection of both these topologies (p=0.05 and 0.01 respectively). Addition of a second geographically distantly sampled conspecific individual to the analysis for five of the six species used resulted in recovery of the tree favouring the basal position of the Ross Seal (*O. rossii*) within the lobodontines, however the SH test and SOWH tests could not reject alternative topologies (Table 2-5c).

The topology of the neighbor-joining minimum evolution tree constructed using the maximum likelihood parameters of the GTR + I + Γ sequence evolution model was identical to that found using ML and Bayesian techniques. Pair-wise divergence measures between all sampled members of the Phocidae ranged from a low of 0.0238 between the species (*P. vitulina* and *P. largha)* of the genus *Phoca* (*sensu stricto*) to an extreme of 0.2687 between the northern species *Halichoerus grypus* and the southern species *Mirounga angustirostris*. The mean of the distribution was 0.1946 and was skewed towards higher values (41 and 64 values were lower and higher than the mean respectively). Based on the accepted taxonomy presented in Table 2-1, pair-wise divergence values between species were subdivided into four categories: 1) species within the same genus, 2) species from different genera within the same tribe, 3) species from different tribes within the same subfamily, and 4) species from different subfamilies within the family Phocidae (Figure 2-3). Because of the contentious issues regarding the status of the genera and their constituent species, comparisons among the five sampled members of the Phocini were excluded from this subdivided comparison, except for the

accepted species relationship of *P. vitulina* and *P. largha*. Each distribution was discrete and non-overlapping, except that the highest value between species from different tribes (*Erignathus barbatus – Phoca vitulina*) within subfamilies was larger than the smallest obtained for comparisons between species from different subfamilies (*Erignathus barbatus – Leptonychotes weddellii*). The distance between the two monk seal species (*Monachus schauinslandi* and *M. monachus*) was 0.1815, much larger than any other species within genus comparisons, falling within the distribution of tribal level differences (0.1357-0.2032). Although not overlapping with any of the other distributions, the pair-wise distance between the Weddell and leopard seals (0.0667) was much lower than other intergeneric comparisons.

Using the distance distributions for each of the taxonomic ranks (species, genus, tribe, and subfamily) established above, we compared the agreement of three frequently encountered taxonomic classifications within the Phocini (Figure 2-3a-c). A taxonomic scheme including only two genera, *Halichoerus* and *Phoca* (Burns and Fay, 1970), poses several problems. The distribution of generic differences between *Halichoerus* and *Phoca* spp. overlaps with the values of pair-wise divergence within species of *Phoca*. Generic comparisons using this taxonomy are not only equal in magnitude to species differences within *Phoca* (except for comparisons involving the harp seal) but are also equal to species differences between species of the genus *Mirounga* (Figure 2-3a). Illustrating another inconsistency of this taxonomic approach is the fact that the species differences between the harp seal (*Phoca groenlandica* under this scheme) and the other members of *Phoca* lie in the upper end of the distribution of intergeneric differences. Another taxonomy, currently accepted by the ICZN (International Commission of Zoological Nomencalture), that includes five genera within the Phocini (*Phoca*, *Pusa*, *Halichoerus*, *Pagophilus*, and *Histriophoca*) was examined for its ability to resolve the overlap between the distributions of species and generic differences observed within the Phocini as described above (Figure 2-3b). Although this classification does reduce the overlap between interspecies and intergeneric distributions, it results in an overlap of the intergeneric differences within the Phocini with interspecies differences within the *Mirounga* genus. Therefore a third classification including only the genera *Pagophilus*, (*P. groenlandica*) and *Phoca* (*P. vitulina*, *P. largha*, *P. hispida*, and *P. grypus*) was

examined (Figure 2-3c). This classification resolves all overlap within the Phocini and does not conflict with the species difference observed between *M. leonina* and *M. angustirostris*.

Discussion

Due to their extensive morphological adaptations to an aquatic existence, the Pinnipedia have, in the past, been classified as an order separate from the remaining terrestrial or fissiped carnivores (Scheffer, 1958), or more commonly as a suborder within the Carnivora (King, 1983). However, the notion of a diphyletic origin of the group has argued against the recognition of a distinct Pinnipedia altogether (Mitchell and Tedford, 1973). The analysis presented here places a strongly supported, monophyletic Pinnipedia within the Arctoidea, as advocated by current classifications (McKenna and Bell, 1997), and by recent molecular studies examining higher-level relationships within carnivores (Árnason *et al.* 2002; Lin *et al.*, 2002) and mammals (Ledje and Árnason, 1996). However as noted previously, the Caniformia consists of at least five major lineages, currently granted different taxonomic ranks, yet displaying similar evolutionary divergence levels (Ledje and Árnason, 1996). Although not the intended focus of this study, the topology of the inferred tree presented here reinforces the need for a reassessment of higher-level taxonomy in the Caniformia, especially with respect to the rank afforded to Pinnipedia.

In contrast to the lack of study focusing on the relationships of the major caniform lineages is the substantial attention given to the debate over a monophyletic versus diphyletic origin of the pinnipeds, the identification of the sister group of the pinnipeds (if monophyletic), and the related debate concerning the relationships among the three pinniped families, Phocidae, Odobenidae, and Otariidae. Proponents of a diphyletic origin of the Pinnipedia have dismissed characters inferred as adaptations to an aquatic environment as convergent, and using other non-aquatic characters have suggested that the phocids evolved from a mustelid ancestor, while the otariids and odobenids arose from an ursid lineage (Mitchell and Tedford, 1973). Without the exclusion of characters deemed to be adaptations to a marine environment, most research has supported the idea

of a monophyletic Pinnipedia (Wyss, 1987). However, in this scenario an Odobenidae – Phocidae clade that is the sister group of the Otariidae is supported (Wyss, 1987). In the context of an increasing acceptance of the monophyletic origin of the pinnipeds, considerable debate still exists concerning their affinities. Proponents of the monophyly scenario have most commonly proposed an ursid sister group relationship (Wyss 1987, 1988; Flynn, 1988; Lento *et al.* 1995), however some evidence supports evolution from a mustelid stock (Árnason and Widegren, 1986), and in one case a procyonid (raccoon) affiliation has been proposed (Árnason, 1977). In addition, some research suggests that the question of pinniped relationships involves an unresolvable polytomy (deJong, 1982; Flynn, 1988), and that the question of pinniped affinities should continue to be regarded as uncertain.

Although the inclusion of appropriate outgroups is usually required for the determination of character polarities in a cladistic analysis (Hennig, 1966), the continuing uncertainty regarding pinniped ancestry is unlikely to affect character polarities in this analysis, given that both hypothesized ancestors were included in the analysis which was rooted with the further outgroups, domestic dog (Family Canidae) and domestic cat (Family Felidae: Suborder Feliformia). However the observed placement of the Pinnipedia within a strongly supported Arctoidea, separate from the Cynoidae (which comprises a single family, Canidae), allowed for the analysis to dictate the relationships within the Arctoidea. Thus this study may allow for a fairer assessment of outgroup relationships and character polarities than previous studies that have either not explicitly stated an outgroup, or that have *a priori* selected either an ursid or mustelid outgroup.

No evidence for a diphyletic origin of the Pinnipedia was found in this study even though such an arrangement could have been observed, given the inclusion of members of both ursids and mustelids in the analysis. Alternative phylogenetic methodologies supported contrasting topologies with respect to pinniped ancestral affinities. Maximum parsimony analysis weakly supported a mustelid sister group of the pinnipeds, with non-parametric bootstrap support of 58%. An ursid sister group relationship was the least supported arrangement with a bootstrap value of 18%. In contrast to current thinking, intermediate support (21% of bootstrap trees) was found for an ursid – mustelid clade as sister to the pinnipeds. Support for such an arrangement contrasts with the contention

that either Ursidae or Mustelidae, but not a common ursid – mustelid clade, is the sister group of the Pinnipedia. Maximum likelihood, Bayesian, and neighbor joining methods all supported a sister group relationship between Ursidae and Pinnipedia as the optimal arrangement (Figure 2-2), albeit with differing levels of support. Although the majority of support using these methods indicated that ursids and phocids are sister groups, it was not possible to exclude either a mustelid clade or a common ursid – mustelid clade as the sister groups of the pinnipedia using the SH test. The SOWH test of alternative topologies, performed with a pruned taxon set, suggested rejection of both competing topologies (Table 2-4a). However due to the intense computational requirements of the SOWH test, the partial optimization of the test was used here, and it is therefore unclear whether the full optimization of the test would also result in rejection of the alternative topologies (Goldman *et al.*, 2000). Additionally, recent evidence from empirical data sets has suggested that the SH test is conservative and tends to be prone to acceptance of the null hypothesis even though it may be false while the SOWH test is prone to rejection of the null hypothesis even though it may be true (Buckley, 2002). Taken as a whole the data presented here suggest slightly more support for a sister groups relationship of the ursids and pinnipeds. However as advocated by previous authors this conclusion may be "irrelevant, as the ursids and mustelids may not have truly existed at the time of pinniped divergence. Thus, the whole question of pinniped ancestry may form part of an unresolvable polytomy" (Bininda-Emonds and Russell, 1996).

Notwithstanding the weak corroboration for any particular sister group arrangement, relationships within the Pinnipedia, were generally strongly supported (Figure 2-2). Within a monophyletic Pinnipedia, an Odobenidae – Otariidae clade was strongly supported as sister to the Phocidae. This arrangement has been found in other phylogenetic investigations using DNA sequence data (Perry *et al.*, 1995; Árnason *et al.*, 1995), and suggests that some aquatic adaptations may have evolved in parallel in phocids and odobenids. In addition, relationships within the Otariidae supported the recent contention that the traditional classification of this family into subfamilies Arctocephalinae and Otariinae is not justified (Wynen *et al.*, 2001).

Within the Phocidae, robust support was found for the traditionally accepted subfamily Phocinae. Although the monophyly of Phocinae is universally accepted,

previous studies using both morphological (Bininda-Emonds and Russell, 1996) and molecular (Árnason *et al.* 1995) data have found limited levels of differentiation within this group, resulting in a restricted ability to conclusively resolve internal relationships. One point of almost universal agreement is the recognition of *Erignathus* as a deepest branching phocine, and most grant it a basal position in the phocine radiation. The results presented here support the placement of the bearded seal as the sister group of the other phocines and strongly contradict the recent findings of Bininda-Emonds and Russell (1996) who proposed a more nested position of *Erignathus* within the Phocini. The distinction between *Erignathus* and species in the Phocini and Cystophorini, were greater than other tribal differences exemplifying the large difference between the bearded seal and the remaining phocines.

Among the remaining "northern" seals much more limited differentiation was observed. The position of *Cystophora* (tribe Cystophorini) as sister to the Phocini (including the genera *Phoca*, *Pusa*, *Halichoerus* and *Pagophilus*) was strongly supported. However, the distinction between Cystophorini and the members of the Phocini was the smallest observed between tribes and may explain the limited support found for this topology in Árnason *et al.* (1995). In this study, two major lineages were apparent within the Phocini. The observed position of *Pagophilus* as the sister group sister of the remaining Phocini species is in contrast to studies using small amounts of sequence data (Mouchaty *et al.*, 1995; Perry *et al.*, 1995) and analysis of skeletal morphology (de Muizon, 1982) that suggest an affiliation of *Pagophilus* and *Histriophoca* with *Cystophora*. It also contrasts with other views of the Phocini that include *Pagophilus* and *Histriophoca* as subgenera within *Phoca* based on cranial morphology (Burns and Fay, 1970; Wyss, 1988), or alternatively as species within Phoca without any subgeneric division (Doutt, 1942). As our analysis did not include *Histriophoca fasciata* (ribbon seal) it is unclear how its addition may affect the relationship of *Pagophilus* and *Cystophora*. However, evidence from a single mitochondrial gene (Árnason *et al.*, 1995) supported a *Pagophilus – Histriophoca* clade as sister to the remaining species of the Phocini to the exclusion of *Cystophora* (although with weak support). The inferred sequence divergence between *Pagophilus* and the remaining Phocini is at the level of generic distinction observed within the Lobodontini, and would argue for the recognition

of *Pagophilus* as a distinct genus within the Phocini. Again, due to the omission of *Histriophoca* from the analysis presented here, any comments on the validity of its generic status are precluded.

The division of the Phocini into two clades, along the lines of a *Pagophilus – Histriophoca* clade and a *Phoca – Pusa – Halichoerus* clade, is reminiscent of a previous classification of the group that included two subtribes, Histriophocina and Phocina (Chapskii, 1955). However, the distinction between these two clades was not apparent in studies of cranial morphology (Burns and Fay, 1970), which supported demotion of the four genera, *Phoca*, *Pusa*, *Pagophilus*, and *Histriophoca* to subgeneric status within a newly defined *Phoca*. *Halichoerus* was deemed to have sufficient differentiation to retain its generic status. This, and the fact that *Halichoerus grypus* was the first species to be separated from the all encompassing true seal genus *Phoca* (Scheffer, 1958), has led to the continued recognition of the genus in spite of recent molecular evidence. Sequence divergence between the complete mitochondrial DNA molecules of the ringed, grey, and harbor seals suggests that the three taxa are genetically equidistant (Árnason *et al.*, 1996). Estimated divergences from the mitochondrial cytochrome b gene confirm this conclusion and also suggest that *Halichoerus* is closer to both *Phoca* and *Pusa* than either of these genera are to *Pagophilus* or *Histriophoca* (Árnason *et al.*, 1995). Therefore evidence from molecular phylogenetic studies would support the generic status of both *Pagophilus* and *Histriophoca*, while suggesting that the long standing generic status of *Halichoerus* is not warranted.

Resolution among *Phoca*, *Pusa*, and *Halichoerus* was limited in this study, except for the strong affiliation of *Phoca vitulina* and the recently recognized species *Phoca largha* (Shaughnessy and Fay, 1977). Although all the phylogenetic methodologies used agreed on a *Pusa* - *Halichoerus* clade as sister to *Phoca* spp., nearly equal support was found for a *Phoca – Halichoerus* grouping with *Pusa* as the sister group. However, the contrasting topology, which would be in agreement with the traditional distinction of the grey seal, with *Halichoerus* as sister to the remaining species, was only weakly supported. Although none of the competing topologies could be excluded as equally good explanations of the data with the SH test, a full optimization of the SOWH test suggested rejection of the topology representing *Halichoerus* as sister to a *Pusa* - *Phoca*

grouping. These results generally support the recent contentions that the generic status of *Halichoerus* is not justified unless *Pusa* is also recognized (Árnason et al, 1995, Perry *et al.*, 1995). However pair-wise sequence divergence between *Phoca*, *Pusa*, and *Halichoerus* is not greater than that observed between the species of the genus Mirounga, arguing that the generic status of *Halichoerus* and *Pusa* should, more appropriately, be abolished altogether, thus including the grey seal and ringed seal as members of the genus *Phoca*.

The composistion of the subfamily Monachinae has fluctuated over its taxonomic history. Due to the general lack of distinctiveness between *Monachus* spp. and members of the Lobodontini, Scheffer (1958) joined the antarctic seals with the monk seals in a newly defined Monachinae. With the addition of *Mirounga* spp., following the division of the Cystophorinae (King, 1966), the Monachinae now encompass all "southern" seals. Most authors agree that the monk seals (*Monachus* spp.) are early branching extant phocid species and that the genus is monophyletic. In addition, it has been suggested that, disregarding the extreme geographic distribution of the group, the differences between the species are so slight that they could be considered as subspecies of a single species (Scheffer, 1958). However, it has recently been suggested that *Monachus* may be paraphyletic (Wyss, 1988), and owing to the primitive nature of the monk seals they should be placed ancestral to all other phocids (Repenning and Ray, 1972). This view would hold the Monachinae paraphyletic and would require a reevaluation of phocid taxonomy. The analysis presented here indicates strong support for the traditional Monachinae, and for a monophyletic *Monachus* as the basal split in the "southern" radiation. The large divergence between the two sampled species of monk seal indicated that both *Monachus monachus* and *Monachus schauinslandi* are ancient lineages that diverged soon after the origin of their monachine ancestor. This arrangement would argue against any connotations of subspecific relationships within *Monachus*, a fact indicated by the sequence divergence between species being as large as tribal differences within the Phocidae as a whole. The relationship of the recently extinct species *Monachus tropicalis* (Caribbean monk seal) to the other monk seals could not be assessed, as it was not included in this study. However, the position of *Monachus* within the subfamily Monachinae illustrates why doubts regarding the monophyly of the

subfamily have recently been raised. The relatively short branch separating the inferred *Monachus* common ancestor from the node leading to the other Monachine lineages and the relatively short period of evolution between the ancestor of *Monachus* and the ancestor of the Phocidae as a whole may restrict phylogenetic resolution at the base of monachine radiation.

Due to potential paraphyly and the fluctuating membership of the Monachinae, the use of tribal distinctions within the subfamily has been questioned (Henday and Repenning, 1972). Despite this, de Muizon (1982) divided the Monachinae into the three tribes Monachini (monk seals), Miroungini (elephant seals) and the Lobodontini (antarctic seals). The validity of this approach has not been adequately tested. Estimates of sequence divergence between these three Monachine tribes are intermediate to the differences between accepted tribes within the Phocinae, falling above the difference between the Cystophorini and the Phocini, and below the difference between Erignathini and the other Phocinae tribes. Therefore, given the acceptance of tribal definitions within the Phocinae, tribal designations within the Monachinae should also be recognized.

Relationships within the Lobodontini are poorly understood as they have been generally neglected in molecular studies and also because the group has undergone a putative recent rapid radiation such that species are not clearly differentiated from each other. The pairing of *Lobodon* and *Hydrurga* as sister to *Leptonychotes* and *Ommatophoca* has been proposed based on cranial morphology (Hendey, 1972). In contrast to the relationships inferred from morphology, the results presented here strongly suggest an affiliation of *Leptonychotes* and *Hydrurga*. Outside of the strong support for this grouping, resolution within the lobodontines is limited, and competing topologies cannot be ruled out using statistical tests. The addition of sequence data from three other mitochondrial regions statistically supports the placement of *Lobodon carcinophagus* at the base of the lobodontines, although it is unclear whether this is due to the additional information or due to the use of a largely restricted taxon set to perform the statistical test. Analogous to the relationships among *Halichoerus*, *Pusa* and *Phoca*, relationships among genera within the Lobodontini, with the exception of a *Hydrurga – Leptonychotes* grouping, are uncertain and are considered here to be unresolved.

The use of complete mitochondrial genomes in phylogenetic inference has, in general, been restricted to studies addressing higher-level systematic questions (Árnason *et al.* 2002, Lin *et al.* 2002). The strength of the phylogenetic hypothesis presented here indicates that large molecular datasets may also aid in resolving questions at lower taxonomic levels. Although support for the inferred topology presented here is generally very robust, three regions remain unresolved, all occurring in areas of the tree where rapid radiations are thought to have occurred. Even with the large number of informative characters examined here, very few changes occur along these short evolutionary branches, confounding inference of the correct tree with regard to pinniped ancestral affinities, and relationships within the Phocini and Lobodontini. Inference of the correct topology in these cases may require extensive molecular datasets, consisting of the sequences of multiple nuclear genes, or possibly information on chromosomal rearrangements (Nie *et al.*, 2002) or retroposon insertions (Shedlock and Okada, 2000).

Despite an inability to effectively resolve the aforementioned rapid radiation polytomies, it should be noted that the phylogenetic hypothesis presented here is in general agreement with most recent studies, and does not introduce any novel, or surprising, groupings not previously observed. It presents a further refinement of phocid taxonomy, supporting many recent hypotheses, such as the evaluation of taxonomic relationships within the Phocini, and provides evidence for the use of tribal distinctions within the monachines. In contrast, the phylogeny presented here refutes other recent hypotheses concerning Phocid evolution, including the proposed paraphyly of Monachinae and of the genus *Monachus*. As such, the phylogeny presented here may be used to reevaluate the characters used to support alternative hypotheses, and in light of the fossil record, will be valuable in studies examining the historical biogeography of the phocids.

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Table 2-1. Taxonomy of the Phocidae. [1] Indicates species not included in this study. [2] Tribal distinctions within the subfamily Monachinae are contentious. ‡ Indicates species is extinct.

	Common Name	Authority
Class Mammalia		Linnaeus, 1758
Order Carnivora		Bowdich, 1821
Suborder Caniformia		Kretzoi, 1943
Superfamily Arctoidea		Flower, 1869
Family Phocidae		Gray, 1821
Subfamily Phocinae		Gray, 1821
Tribe Erignathini		Chapskii, 1955
Genus *Erignathus*		Gill, 1866
Erignathus barbatus	Bearded seal	Erxleben, 1777
Tribe Cystophorini		Burns and Fay, 1970
Genus *Cystophora*		Nilsson, 1820
Cystophora cristata	Hooded seal	Erxleben, 1777
Tribe Phocini		Gray, 1821
Genus *Phoca*		Linnaeus, 1758
Phoca vitulina	Harbor seal	Linnaeus, 1758
Phoca largha	Spotted seal	Pallas, 1811
Genus *Pusa*		Scopoli, 1777
Pusa hispida	Ringed seal	Schreber, 1775
Pusa caspica [1]	Caspian seal	Gmelin, 1788
Pusa siberica [1]	Baikal seal	Gmelin, 1788
Genus *Halichoerus*		Nilsson, 1820
Halichoerus grypus	Grey seal	Fabricius, 1791
Genus *Pagophilus*		Gray, 1844
Pagophilus groenlandica	Harp seal	Erxleben, 1777
Genus *Histriophoca*		Gill, 1873
Histriophoca fasciata [1]	Ribbon seal	Zimmerman, 1783
Subfamily Monachinae		Trouessart, 1897
Tribe Monachini [2]		Scheffer, 1958
Genus *Monachus*		Fleming, 1822
Monachus monachus	Mediterranean monk seal	Hermann, 1779
Monachus schauinslandi	Hawiian monk seal	Matchie, 1905
‡ *Monachus tropicalis* [1]	Caribbean monk seal	Gray, 1850
Tribe Miroungini [2]		de Muzion, 1982
Genus *Mirounga*		Gray, 1827
Mirounga angustirostris	Northern elephant seal	Gill, 1866
Mirounga leonina	Southern elephant seal	Linnaeus, 1758
Tribe Lobodontini [2]		Scheffer, 1958
Genus *Lobodon*		Gray, 1844
Lobodon carcinophagus	Crabeater seal	Hombron and Jacquinot, 1842
Genus *Ommatophoca*		Gray, 1844
Ommatophoca rossii	Ross seal	Gray, 1844
Genus *Leptonychotes*		Gill, 1872
Leptonychotes weddellii	Weddell seal	Lesson, 1826
Genus *Hydrurga*		Gistel, 1848
Hydrurga leptonyx	Leopard seal	Blainville, 1820

Table 2-2. Genbank accession numbers for novel sequences obtained for this study. Sequences from previous studies are in parentheses.

Species	Gene ND1	ND2	COX I	COX II	ATP8	ATP6
Erignathus barbatus (Bearded seal)	AY377356	AY377276	AY377143	AY377166	AY377189	AY377305
Cystophora cristata (Hooded seal)	AY377357	AY377277	AY377144	AY377167	AY377190	AY377306
Phoca largha (Spotted seal)	AY377360	AY377280	AY377147	AY377170	AY377193	AY377309
Pusa hispida (Ringed seal)	AY377359	AY377279	AY377146	AY377169	AY377192	AY377308
Pagophilus groenlandica (Harp seal)	AY377358	AY377278	AY377145	AY377168	AY377191	AY377307
Monachus monachus (Mediterraean monk seal)	AY377355	AY377275	AY377142	AY377165	AY377188	AY377304
Monachus schauinslandi (Hawiian monk seal)	AY377354	AY377274	AY377141	AY377164	AY377187	AY377303
Mirounga angustirostris (Northern elephant seal)	AY377352	AY377272	AY377138 AY377139	AY377161 AY377162	AY377184 AY377185	AY377300 AY377301
Mirounga leonina (Southern elephant seal)	AY377353	AY377273	AY377140	AY377163	AY377186	AY377302
Lobodon carcinophagus (Crabeater seal)	AY377348	AY377268	AY377130 AY377131	AY377153 AY377154	AY377176 AY377177	AY377292 AY377293
Ommatophoca rossii (Ross seal)	AY377349	AY377269	AY377132 AY377133	AY377155 AY377156	AY377178 AY377179	AY377294 AY377295
Leptonychotes weddellii (Weddell Seal)	AY377351	AY377271	AY377136 AY377137	AY377159 AY377160	AY377182 AY377183	AY377298 AY377299
Hydrurga leptonyx (Leopard seal)	AY377350	AY377270	AY377134 AY377135	AY377157 AY377158	AY377180 AY377181	AY377296 AY377297
Odobenus rosmarus (Walrus)	AY377361	AY377281	AY377148	AY377171	AY377194	AY377310
Arctocephalus australis (South American fur seal)	AY377363	AY377283	AY377150	AY377173	AY377196	AY377312
Otaria byronia (South American sea lion)	AY377362	AY377282	AY377149	AY377172	AY377195	AY377311
Gulo gulo (Wolverine)	AY377364	AY377284	AY377151	AY377174	AY377197	AY377313
Mustela vison (Mink)	AY377365	AY377285	AY377152	AY377175	AY377198	AY377314

Table 2-2. *Continued*

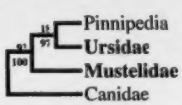
Species	Gene COX III	ND3	ND4L	ND4	ND5	CYTB
Erignathus barbatus (Bearded seal)	AY377258	AY377212	AY377235	AY377338	AY377375	(X82295)
Cystophora cristata (Hooded seal)	AY377259	AY377213	AY377236	AY377339	AY377376	(X82294)
Phoca largha (Spotted seal)	AY377262	AY377216	AY377239	AY377342	AY377379	(X82305)
Pusa hispida (Ringed seal)	AY377261	AY377215	AY377238	AY377341	AY377378	(X82304)
Pagophilus groenlandica (Harp seal)	AY377260	AY377214	AY377237	AY377340	AY377377	(X82303)
Monachus monachus (Mediterraean monk seal)	AY377257	AY377211	AY377234	AY377337	AY377374	AY378327
Monachus schauinslandi (Hawiian monk seal)	AY377256	AY377210	AY377233	AY377336	AY377373	(X72209)
Mirounga angustirostris (Northern elephant seal)	AY377253 AY377254	AY377207 AY377208	AY377230 AY377231	AY377334	AY377371	AY377325
Mirounga leonina (Southern elephant seal)	AY377255	AY377209	AY377232	AY377335	AY377372	AY377326
Lobodon carcinophagus (Crabeater seal)	AY377245 AY377246	AY377199 AY377200	AY377222 AY377223	AY377330	AY377367	AY377231
Ommatophoca rossii (Ross seal)	AY377247 AY377248	AY377201 AY377202	AY377224 AY377225	AY377331	AY377368	AY377322
Leptonychotes weddellii (Weddell Seal)	AY377251 AY377252	AY377205 AY377206	AY377228 AY377229	AY377333	AY377370	AY377324
Hydrurga leptonyx (Leopard seal)	AY377249 AY377250	AY377203 AY377204	AY377226 AY377227	AY377332	AY377369	AY377323
Odobenus rosmarus (Walrus)	AY377263	AY377217	AY377240	AY377343	AY377380	(X82299)
Arctocephalus australis (South American fur seal)	AY377265	AY377219	AY377242	AY377345	AY377382	AY377329
Otaria byronia (South American sea lion)	AY377264	AY377218	AY377241	AY377344	AY377381	AY377328
Gulo gulo (Wolverine)	AY377266	AY377220	AY377243	AY377346	AY377383	(AB051245)
Mustela vison (Mink)	AY377267	AY377221	AY377244	AY377347	AY377384	(AB026109)

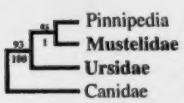
Table 2-2. *Continued*

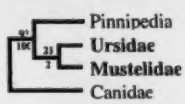
Species	ND6	12S rRNA	16S rRNA
Mirounga angustirostris (Northern elephant seal)	AY377319	AY377290	AY377388
Mirounga leonina (Southern elephant seal)	AY377320	AY377291	AY377389
Lobodon carcinophagus (Crabeater seal)	AY377315	AY377286	AY377384
Ommatophoca rossii (Ross seal)	AY377316	AY377287	AY377385
Leptonychotes weddellii (Weddell Seal)	AY377318	AY377289	AY377387
Hydrurga leptonyx (Leopard seal)	AY377317	AY377288	AY377286

Table 2-3. Maximum parsimony (MP) tree lengths, log likelihood maximum likelihood (ML) scores, likelihood differences between the ML tree and suboptimal trees and results of the SH test for competing topologies concerning a) pinniped sister group relationships, b) relationships within Phocini species, and c) relationships within the Lobodontini using the complete 27 species taxon set. Maximum parsimony bootstrap support above and Bayesian support below branches.

a) 27 Species - pinniped sister group relationships

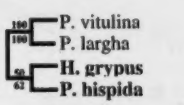


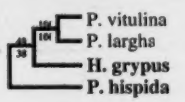


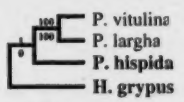


Tree Length (MP)	19770	**19760**	19768
lnL (ML)	**-91756.3545**	-91760.35783	-91760.80254
lnL Difference		4.00332	4.44803
SH Test (p-value)		0.467	0.43

b) 27 Species - Phocini

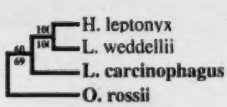


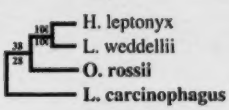


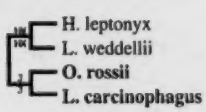


Tree Length (MP)	**19760**	19771	19782
lnL (ML)	**-91756.3545**	-91756.67184	-91761.71162
lnL Difference		0.31734	5.35711
SH Test (p-value)		0.627	0.186

c) 27 Species - Lobodontini



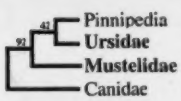
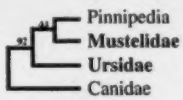
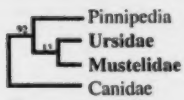




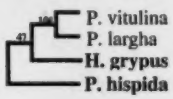
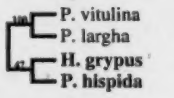
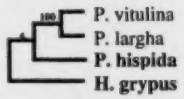
Tree Length (MP)	**19760**	19771	19780
lnL (ML)	**-91756.3545**	-91757.17702	-91759.17048
lnL Difference		0.82251	2.81598
SH Test (p-value)		0.552	0.31

Table 2-4. Maximum parsimony (MP) tree lengths, log likelihood maximum likelihood (ML) scores, likelihood differences between the ML tree and suboptimal trees, and results of the SH and SOWH tests for competing topologies concerning a) pinniped sister group relationships, b) relationships within Phocini species, and c) relationships within the Lobodontini using pruned taxon sets. Maximum parsimony bootstrap above branches.

a) 13 Species - pinniped sister group relationships

			
Tree Length (MP)	**13798**	**13798**	13804
lnL (ML)	**-66732.36543**	-66735.17981	-66736.01552
lnL Difference		2.81438	3.65009
SH Test (p-value)		0.5	0.458
SOWH Test (p-value)			$T_{(0)}$
	$T_{(ML)}$	*< 0.01**	*< 0.01**

b) 7 Species - Phocini

			
Tree Length (MP)	**3127**	**3127**	3134
lnL (ML)	**-28106.37918**	-28106.54402	-28108.66392
lnL Difference		0.16484	2.28474
SH Test (p-value)		0.601	0.3
SOWH Test (p-value)			$T_{(0)}$
	$T_{(ML)}$	0.09	*0.02**

c) 8 Species - Lobodontini

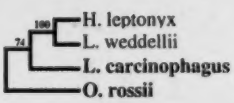
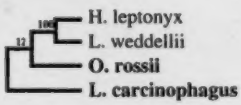
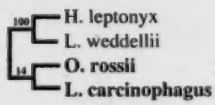
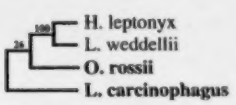
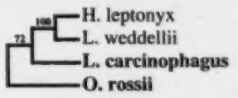
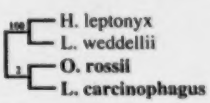
			
Tree Length (MP)	**4342**	4353	4354
lnL (ML)	**-32529.47664**	-32531.55857	-32531.91277
lnL Difference		2.08192	2.43613
SH Test (p-value)		0.25	0.22
SOWH Test (p-value)			$T_{(0)}$
	$T_{(ML)}$	0.05	0.09

Table 2-5. Maximum parsimony (MP) tree lengths, log likelihood maximum likelihood (ML) scores, likelihood differences between the ML tree and suboptimal trees, and results of the SH and SOWH tests for competing topologies using a) 6 species dataset and the original 12 coding regions, b) 6 species dataset and the original 15 coding regions, and c) an additional conspecific individual and a seven gene dataset. Maximum parsimony bootstrap above branches.

a) 6 Species -12 Genes

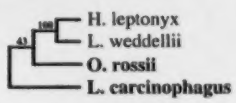


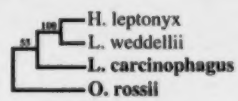


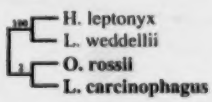


Tree Length (MP)	2776	**2769**	2789
lnL (ML)	**-26613.21792**	-26613.67288	-26614.52049
lnL Difference		0.45496	1.30257
SH Test (p-value)		0.534	0.384
SOWH Test (p-value)		$T_{(0)}$	
	$T_{(ML)}$	0.18	0.13

b) 6 Species -15 Genes

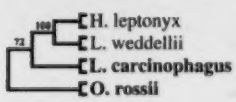


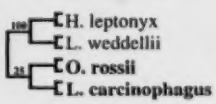


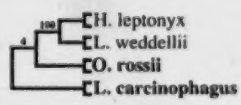


Tree Length (MP)	3204	**3202**	3221
lnL (ML)	**-33065.16842**	-33066.37631	-33066.79954
lnL Difference		1.20789	1.63112
SH Test (p-value)		0.44	0.362
SOWH Test (p-value)		$T_{(0)}$	
	$T_{(ML)}$	*0.05**	*0.01**

c) 6 Species (2 individuals) - 7 Genes







Tree Length (MP)	**1163**	1170	1175
lnL (ML)	**-11300.2716**	-11300.27845	-11300.27864
lnL Difference		0.00685	0.00704
SH Test (p-value)		0.606	0.701
SOWH Test (p-value)		$T_{(0)}$	
	$T_{(ML)}$	0.43	0.2

Table 2-6. Primer names, and locations relative to the harbour seal sequence (*Phoca vitulina*), X63768, Árnason and Johnsson, 1992). A indicates the primer was used to amplify particular fragments of the mtDNA molecule, and S indicates the primer was used as a sequencing primer.

Appendix 1. Primer list.			Crabeater Seal (CR6.3)	Crabeater Seal (C2106)	Ross Seal (RERL)	Ross Seal (R2769)	Leopard Seal (L2677)	Leopard Seal (L3151)	Weddell Seal (W96.8)	Weddell Seal (W12156)	Northern Elephant Seal (2013)	Northern Elephant Seal (N17)	Southern Elephant Seal	Hawaiian Monk Seal	Mediterranean Monk Seal	Bearded Seal	Hooded Seal	Harp Seal	Ringed Seal	Spotted Seal	Walrus	South American Sea Lion	South American Fur Seal	Wolverine	Mink
Primer name	**Location**	**Primer sequence (5' to 3')**																							
mtDNA1 fragment																									
Amplifying primers																									
mtDNA1H	16807-00001	CAA ATG GGA CAT CTC GAT GGA CTA	AS		AS		AS		AS		AS		AS												
mtDNA1L	02361-02382	CAG CTA TCA CCA GGC TCG TTA G	AS		AS		AS		AS		AS		AS												
Internal primers																									
mtDNA1H.int2	00965-00985	CAC TGA AAA TGC CTA GAC GAG	S		S		S		S		S		S												
mtDNA1H.int3	01427-01444	CAA ACT GGG ATT AGA TAC	S		S		S		S		S		S												
mtDNA1L.int2	01427-01444	GTA TCT AAT CCC AGT TTG	S		S		S		S		S		S												
mtDNA2 fragment																									
Amplifying primers																									
mtDNA2H	01915-01940	GGA GAT AAG TCG TAA CAA GGT AAG CA	AS		AS		AS		AS		AS		AS												
mtDNA2L	03782-03802	TCC TAC GAT GTT GGG TCC TTT	AS		AS		AS		AS		AS		AS												
Internal primers																									
mtDNA2H.int	02268-02286	CGA AAC CAG ACG AGC TAC C	S		S		S		S		S		S												
mtDNA2H.int3	03039-03058	GAA ATT GAC CTT CCC GTG AA	S		S		S		S		S		S												
mtDNA2L.int	03283-03300	CCC CAG GGT AAC TTG TTC	S		S		S		S		S		S												
mtDNA2L.int2	02835-02848	GTG TTT GCC GAG TT	S		S		S		S		S		S												
mtDNA3 fragment																									
Amplifying primers																									
mtDNA3H	03450-03471	AAT CCA GGT CGG TTT CTA TCT A	AS		AS		AS		AS		AS		AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA3L	05386-05409	ATC CTA TAT GGG CGA TTG ATG AGT	AS		AS		AS		AS		AS		AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
Internal primers																									
mtDNA3H.int	03818-03831	CAA CCT ATC GCA GA	S		S		S		S		S		S	S			S		S	S					
mtDNA3H.int2	04130-04148	CTA TCA GTC CTA CTA AT															S							S	
mtDNA3H.int2 (mu)	04088-04110	GCC CAA ACA ATC TCA TAC GAA GT	S											S	S	S		S		S		S			S
mtDNA3H.int3	04837-04856	GAA ATA TGT CTG ACA AAA GA	S		S		S		S		S		S	S	S	S	S	S	S	S		S	S	S	S
mtDNA3L.int2	04738-04756	GGT AGC ACG AAG ATT TTT G	S		S		S		S		S		S	S	S	S	S	S	S	S	S	S	S	S	S
mtDNA3L.int3	04196-04217	GTC ATG TAG GGA AAA TTA GTC A	S		S		S		S		S		S	S				S	S		S	S	S	S	
mtDNA3L.int3 (mu)	04130-04152	CCG TTT ATT AGT AAG ACT GAT AG														S	S			S					S
mtDNA4 fragment																									
Amplifying primers																									
mtDNA4H	04813-04837	CGA AAA TGT TGG TTT ATA CCC TTC C	AS		AS		AS		AS		AS		AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA4L	06191-06210	TGC CAA GCT CTG TGG TGA AT																						AS	AS
mtDNA4L (aa)	06094-06114	GGG AGA AGT AGA TTG AAG	AS		AS		AS		AS		AS		AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS		
Internal primers																									
mtDNA4H.int	05348-05361	AGG ACT AAA CCA AAC TCA AC	S		S				S		S		S	S	S	S		S	S	S	S	S	S	S	S
mtDNA4L.int	05914-05935	CAC TTA CTT AGG GCT TTG AAG G	S		S		S		S		S		S	S	S	S	S	S	S	S	S	S	S	S	S
mtDNA5 fragment																									
Amplifying primers																									
mtDNA5H	08967-05993	GGA CTG CAA GAA CAT ATC TCA CAT CAA	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS		AS	AS	A	AS	AS	AS	AS		AS	AS
mtDNA5H (pin)	05811-05836	TCA TCC ACA ACG ACA CTA TCA CTA TC																					A		
mtDNA5H (sk)	06054-06075	CCC ACG AAA ATT TAG TTA ACA G													AS										
mtDNA5L	07751-07776	GGA GGA GGA CAT CCA TGT AGT CAT TC	AS	A	AS	A	AS	A	AS	A	AS	A	AS	AS		AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA5L (sk)	07851-07871	GGT TAT GAC GTT GGC TTG AAA													AS										
Internal primers																									
mtDNA5H (sfa)	06913-06930	GCC TTC AAA GCC CTA AGT																					S		
mtDNA5H.int	06797-06816	GCA ATA TCT CAA TAT CAA AC														S	S		S						
mtDNA5H.int (as)	06616-06635	CCT CCA TAG TAG AAG CAG	S	S	S	S	S	S	S	S	S	S	S	S	S	S		S	S	S				S	
mtDNA5H.int (mu)	06791-06816	CCT CCT GCA ATA TCA CAA TAC CAA AC																S			S	S	S		S
mtDNA5H.int2	07172-07196	GAC ACA CGA GCT TAC TTC ACT TCA G	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
mtDNA5L.int	06980-06993	TGT CCG AAG AAT CA												S	S		S		S			S	S		
mtDNA5L.int (as)	07175-07193	AAG TAA AGT ATG CTC GTG T	S		S		S		S		S					S	S	S	S	S	S	S		S	S
mtDNA5L.int (sfa)	06854-06872	GCA GGG ATA GCA GAA GTA G																					S		
mtDNA5L.int2 (as)	06626-06641	CTG CAC CTG CTT CTA C	S		S		S		S		S		S	S	S	S		S	S	S				S	

Table 2-6. *Continued*

Appendix 1. Primer list			Crabeater Seal (CR6.3)	Crabeater Seal (C2106)	Ross Seal (RERL)	Weddell Seal (WW6.6)	Northern Elephant Seal (2013)	Northern Elephant Seal (N17)	Southern Elephant Seal	Hawaiian Monk Seal	Mediterranean Monk Seal	Bearded Seal	Hooded Seal	Harp Seal	Ringed Seal	Spotted Seal	Walrus	South American Sea Lion	South American Fur Seal	Wolverine	Sea
Primer name	**Location**	**Primer sequence (5' to 3')**																			
mtDNA6 fragment																					
Amplifying primers																					
mtDNA6H	07644-07668	GCT CAT TTA TTT CAC TAA CAG CAG T	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA6H (ses)	07415-07437	CTA TCA ATA GGA GCA GTC TTC GC							A												
mtDNA6U (ly)	07769-07794	CCT CCT CCA TAT CAC ACA TTT GAA GA									AS										
mtDNA6L	09475-09496	GGG CTA CAG CAA ATT CAA GGA T	AS	A	AS	A	AS	A	AS	A	AS	A		AS		AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA6L (ses)	09544-09564	GGT GAG TCA TTA GGT GTT ATC							A												
mtDNA6L (ly)	09780-09799	CCG TAT CGT AGT CCT TTT TG									AS										
Internal primers																					
mtDNA6H (ses2)	07642-07660	AGG TTC ATT TAT CTC ACT T							A												
mtDNA6H.int	07849-07870	GGT TTC AAG CCA ATA TCA TAA C	S	S	S	S	S	S	S	S	S	S		S	S	S	S	S	S	S	S	S	S	S
mtDNA6H.int (ses)	08097-08116	ACC ACA AAA CTA ACC CAC AC							S												
mtDNA6H.int2	08334-08351	CAA GAA CTA AAG CCT GGA														S		S			
mtDNA6H.int2 (mu)	08409-08431	CGC ATA CTA ATC TCG TCC GAA GA											S	S			S			S	S
mtDNA6H.int2 (as)	08478-08493	GCC ATT CCA GGA CGA C	S	S	S	S	S	S	S	S	S	S	S		S	S	S		S	S	
mtDNA6H.int3	08861-08881	CCT TGA GAA GAA AAA TGA ACG	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
mtDNA6L (ses2)	09435-09452	AAA GTG ATT GAG GCT GTA G							S												
mtDNA6L.int	09241-09263	GGT AGA AAG TGA GCC AAG GAT GC																	S	S	S
mtDNA6L.int (ses)	08888-08907	GTA GCG AAA GAG GTG AAT A							S												
mtDNA6L.int2	08861-08881	CGT TCA TTT TTC TTC TCA AGG	S		S		S		S		S	S	S	S	S	S	S			S	S
mtDNA6L.int3	08266-08281	TCG TAA CTT CAA TAT CA														S				S	
mtDNA6L.int3 (as)	08286-08304	GGT CTT CGT AGT CTG TGT A	S		S		S		S		S	S	S	S	S		S	S			S
mtDNA6L.int3 (ses)	08196-08216	TAT GTA AAG GAT TCG TAA TGA							S		S										
mtDNA7 fragment																					
Amplifying primers																					
mtDNA7H	09247-09269	TTG GCT CAC TTT CTA CCT CAA GG	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA7L	11109-11135	GTG GGG ATG ATG ATT TTT AGC ATT GTA	AS	A	AS	A	AS	A	AS	A	AS	A	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
Internal primers																					
mtDNA7H.int	09861-09877	CAC TCA AGC CTA GCA CC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
mtDNA7H.int (af)	09853-09874	GCC TTT TAT CAC TCA AGC CTA GC																		S	
mtDNA7H.int2 (as)	10535-10550	CAT AGG ATC AGC ACG C	S	S	S	S	S	S	S	S	S	S	S				S	S	S	S	S	S	S	
mtDNA7L.int	10602-10620	GTA GTG CAA TTT CTA GGT C											S	S	S	S				S	S	S	S	
mtDNA7L.int (as)	10539-10553	TAG GCG TGC TGA TCC	S		S		S		S		S	S	S	S	S	S	S	S	S	S	S			
mtDNA7L.int (mu)	10805-10827	GGT AGG AGG AGT GCG ATT TCT AG																						S
mtDNA7L.int2 (mu)	09867-09883	GGG GTT GGT GCT AGG CT																		S	S	S	S	S
mtDNA7L.int2 (as)	09900-09916	ATG CCT GTG GGT GGT CA	S		S		S		S		S	S	S	S	S	S	S	S	S			S		
mtDNA8 fragment																					
Amplifying primers																					
mtDNA8H	10770-10792	CCA AAA CAA ATG ATT TCG ACT CA	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA8L	12632-12658	GGT TCC TAA GAC CAA TGG ATT ACT TCT	AS	A	AS	A	AS	A	AS	A	AS	A	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
Internal primers																					
mtDNA8H.int	11192-11207	CAA CAG CTT ACA GCC T										S			S						
mtDNA8H.int (as)	11437-11452	ACA TTC ACC GCC ACA G	S	S	S	S	S	S	S	S	S	S	S	S	S	S			S	S	S			
mtDNA8H.int (mu)	11102-11123	AAT CTA CTA CAA TGC TAA AAA T												S		S		S				S	S
mtDNA8H.int2	11725-11744	GTA AAA ATA CCT CTA TAC GG							S					S	S	S	S	S		S		S		
mtDNA8H.int2 (as)	12060-12085	CAG CCC TAA TAA TCG C	S	S	S	S	S	S	S	S	S	S		S	S			S	S	S	S	S		S
mtDNA8L.int (as)	12256-12274	ATG AAA ATG AGG CTA TTA C	S		S		S		S		S			S	S	S	S	S		S				
mtDNA8L.int2	12041-12060	TAT TAA GGC TGT TGC TCC TA							S	S	S	S	S	S	S	S						S	S
mtDNA8L.int3	11725-11744	CCG TAT AGA GGT ATT TTT AC			S					S		S	S	S	S	S		S				S	S

Table 2-6. *Continued*

Appendix 1. Primer list.			Crabeater Seal (CM13)	Crabeater Seal (C2106)	Ross Seal (RERL)	Ross Seal (RG750)	Leopard Seal (L2677)	Leopard Seal (L3151)	Weddell Seal (W06.6)	Weddell Seal (W12156)	Northern Elephant Seal (p5f12)	Northern Elephant Seal (N17)	Southern Elephant Seal	Hawaiian Monk Seal	Mediterranean Monk Seal	Bearded Seal	Hooded Seal	Harp Seal	Ringed Seal	Spotted Seal	Walrus	South American Sea Lion	South American Fur Seal	Wolverine	Sea
Primer name	**Location**	**Primer sequence (5' to 3')**																							
mtDNA9 fragment																									
Amplifying primers																									
mtDNA9H	12391-12414	AAA CCA TCA TTC ACA CGA GAA AAC	AS		AS		AS		AS		AS					AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
mtDNA9H (seq)	12252-12273	CGT AGT AAT AGC CTC ATT TTC A											AS	AS	AS										
mtDNA9L	13901-13925	GAG TTA GTA ATA GGG CTC AGG CGT T	AS		AS		AS		AS		AS					AS	AS	AS	AS	A	AS	AS	AS	AS	A
mtDNA9L (seq)	13743-13762	TTT TCG GAT GTC TTG TTC AT											AS	AS	AS										
Internal primers																									
mtDNA9H (af)	12475-12493	CTA GGA CCC ATC TAC TGT A														S	S			S					
mtDNA9H.int	12874-12890	CTC CAA ATA AAA GTA AT	S		S		S		S		S		S	S	S	S	S	S	S	S		S	S	S	S
mtDNA9H.int2	13127-13140	GTA GGG ATC ATA TC																						S	
mtDNA9H.int2 (mu)	13405-13428	ACC CCT GTA TCA GCC CTA CT	S						S		S					S	S	S	S	S		S	S	S	S
mtDNA9H.int2 (seq)	13012-13030	GCA CTC AGA CCC TTT CAT C											S		S										
mtDNA9L.int	13883-13902	TTG GTA TAC GAC GTG TTG GC	S		S		S		S		S					S	S	S	S	S			S	S	S
mtDNA9L.int2	13364-13380	GGA TGA AGT CCG AAT TG			S										S	S		S	S						
mtDNA9L.int2 (seq)	13443-13462	GAA GAC CCC TGC TAC TAC TA											S												
mtDNA9L.int3	13115-13135	GAT CCC TAC TCC TTC TCA GCC											S	S	S	S	S	S	S	S	S		S	S	S
mtDNA10A																									
Amplifying primers																									
mtDNA10AH	13408-13431	TAC TCC TGT TTC AGC CCT ACT CCA																					AS		
mtDNA10AH (old)	13595-13617	GCA TTC TCA ACC TCA AGC CAA CT	AS		AS		AS		AS		AS		AS	AS	AS	AS	AS	AS	AS	AS	A	AS			
mtDNA10AH (mu)	13818-13839	GCC TAG CAC TTA CAG GAA TAC C																						AS	AS
mtDNA10AL (old)	15491-15517	CTC AAA ATG ATA TTT GTC CTC ATG GTA	AS		AS		AS		AS		AS		AS	A	AS	A	A	A	A	A	A	A	A		
mtDNA10AL (mu)	14779-14799	GGC TAC TGA GCA GTA TCC TGA																						AS	AS
Internal primers																									
mtDNA10H.int	13898-13915	ACC AAC GCC TGA GCC CTA	S		S		S		S		S		S	S	S				S	S	S	S	S	S	
mtDNA10H.int (pin)	13895-13912	ATA CCA ACG CCT GAG CC												S		S	S	S			S				
mtDNA10H.int2	14095-14081	GGA AGT ATT TTC GCA G	S		S		S		S		S		S		S					S	S	S		S	
mtDNA10H.int3	14581-14600	TAT AAA GCC GCA ATC CCC AT			S		S		S		S										S	S			
mtDNA10L.int3	15422-15440	CCA ATG TTT CAT GTT TCT G	S		S		S		S		S		S		S					S	S	S			
mtDNA10L.int4	14941-14959	CTT CTA AGC CTT CTC CCA T			S		S		S		S				S						S	S			
mtDNA10L.int5	14605-14621	GGG TTT TTT AGT GAG GA	S		S		S		S		S		S	S		S	S	S	S	S	S	S			
mtDNA10B																									
Amplifying primers																									
mtDNA10BH (old)	15278-15302	CCT TCT CAT CAG TAA CCC ACA TCT G	AS		AS		AS		AS		AS		AS		AS							AS	AS		
mtDNA10BL (old)	00103-00125	ATT TGA CTG CGT CGA GAC CTT TA	AS		AS		AS		AS		AS		AS		AS							AS	AS		
Internal primers																									
mtDNA10BH (old2)	15405-15430	CGG CTC CTA CAC ATT CAC AGA GAC AT													S							S			
mtDNA10U.int4	15356-15374	CCA TCT TCT TTA TCT GCC T					S		S																
mtDNA10U.int5	15733-15750	GAC TCA GAC AAA ATC CCA	S		S		S		S		S		S		S							S	S		
mtDNA10BL (old2)	00085-00108	CCT TTA CGG TCA TAG CTG AGT CAT																				S			
mtDNA10L.int	16054-16067	AAT AGG CAT TGG CT																				S			
mtDNA10L.int2	15736-15754	GGA ATG GGA TTT TGT CTG A	S		S		S		S		S		S		S							S	S		
mtDNA11 fragment																									
Amplifying primers																									
mt11H	16095-16120	CTA ACA TGA ATC GGA GGA CAA CCA G	AS		AS		AS		AS		AS		AS												
mt11L	00965-00987	GGC TCA TCT AGG CAT TTT CAG TG	AS		AS		AS		AS		AS		AS												
Internal primers																									
mt11H.int	00661-00673	CAA ACC CCC CTT A	S		S		S		S		S		S												

Figure 2-1. Schematic representation of current problems in phocid systematics. Dark lines show the most commonly supported tree. Dotted lines specify alternative positions supported in some studies. Boxes indicate areas of uncertainty due to poorly resolved ingroup relationships, or possible paraphyly (*Monachus* spp.)

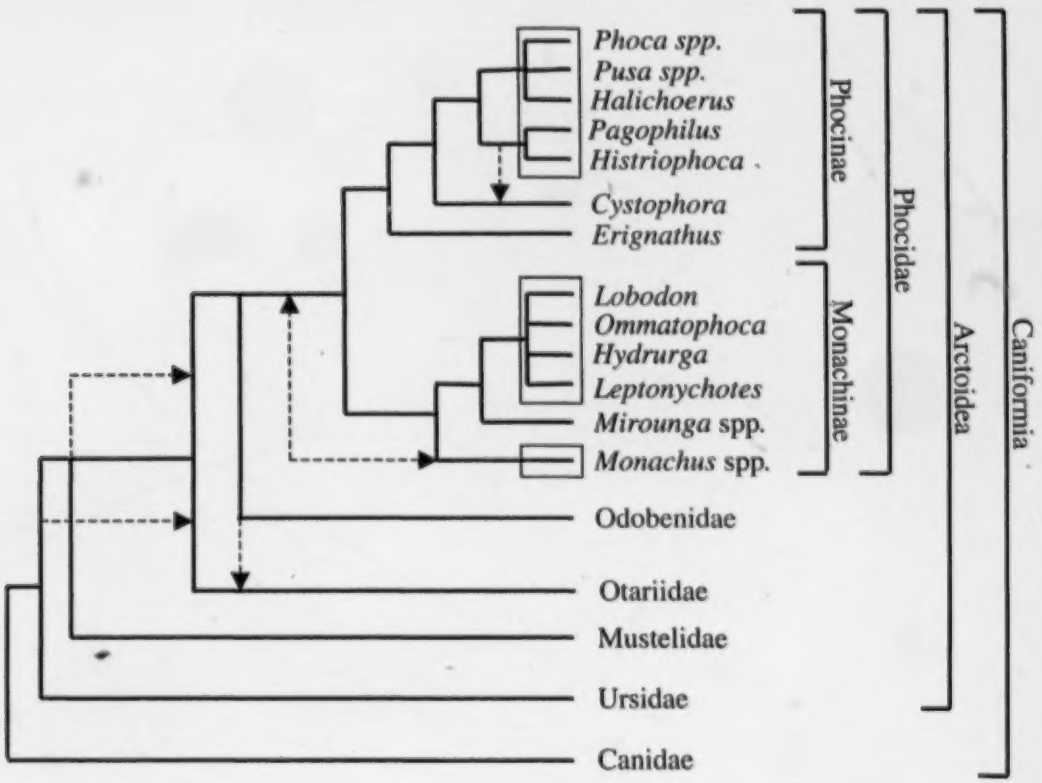


Figure 2-2. Maximum likelihood tree, with optimized branch lengths, recovered using the GTR + I + Γ model. Maximum parsimony (MP) bootstrap support and Bayesian posterior support shown above and below branches respectively. Double headed arrow indicates alternative position of branches seen in the MP tree. Alternative support for competing topologies with respect to the a) lobodontines and b) phocines is shown with the arrangement found in the ML tree shaded.

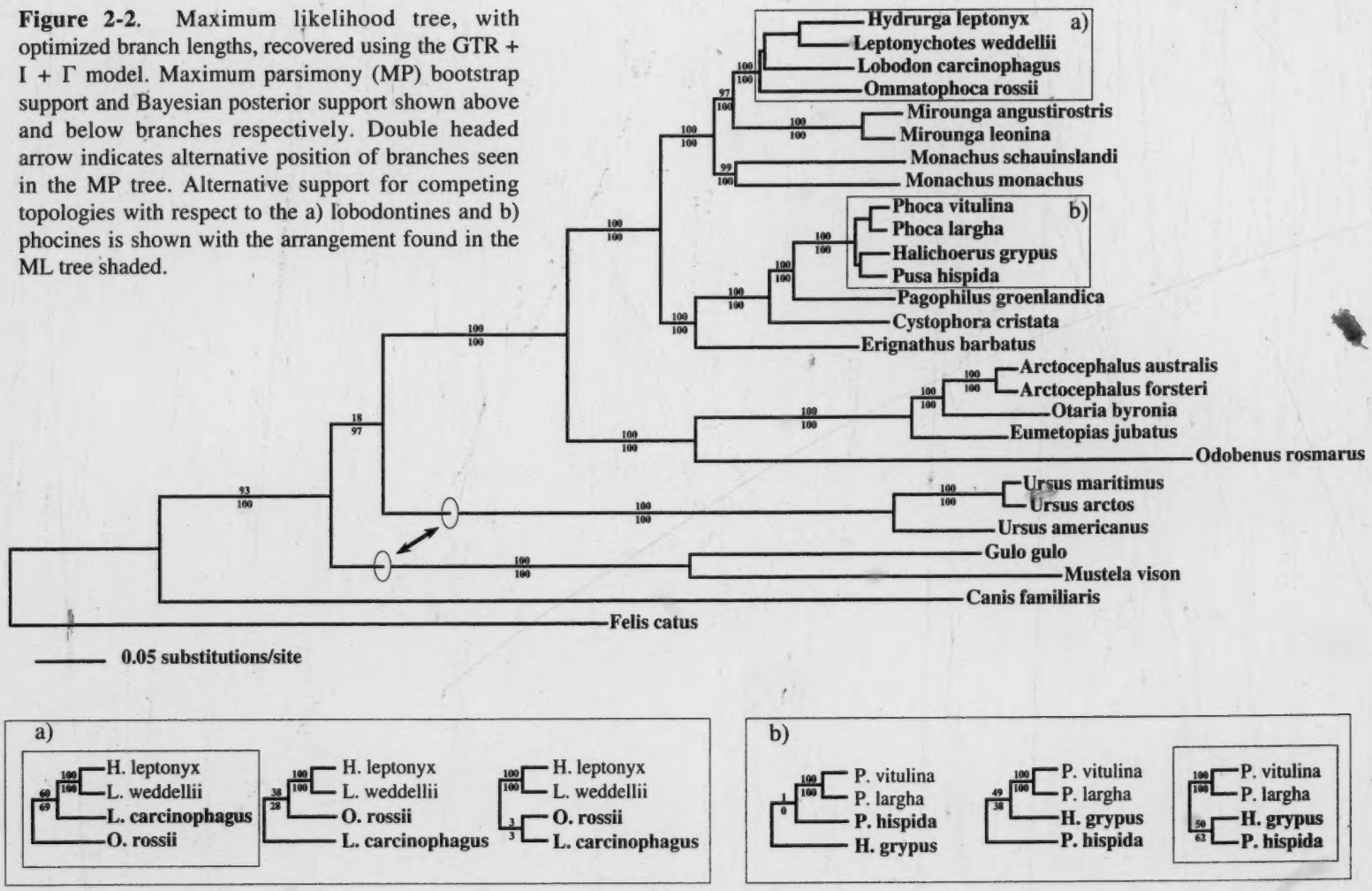
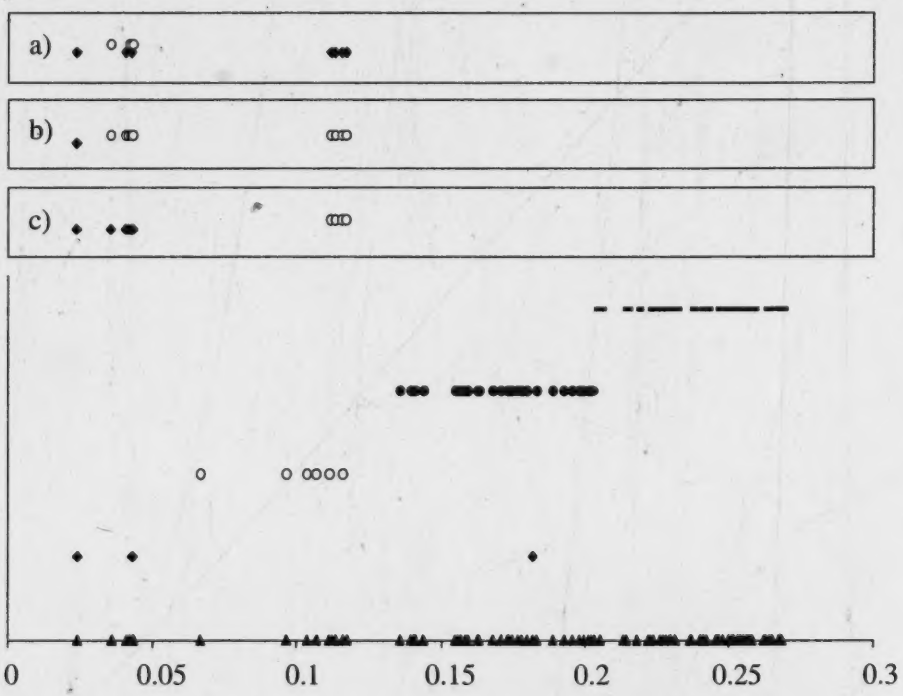


Figure 2-3. Distribution of pair-wise sequence divergence between all phocid species. The distribution of all pair-wise comparisons (filled triangles) is shown along the X axis, and is partitioned into distances between species from the same genus (filled diamonds), species in different genera from the same tribe (open circles), species in different tribes within the same subfamily (filled circles), and species in different subfamilies (dashes). Distances between species within the tribe Phocini are not included in the partitioned distribution. Comparison of pair-wise species and generic divergence based on three taxonomic naming schemes within the Phocini.
a) Two genera; Phoca (groenlandica, hispida, vitulina, largha) and Halichoerus (grypus)
b) Four genera; Phoca (vitulina, largha), Pusa (hispida), Pagophilus (groenlandica), and Halichoerus (grypus)
c) Two genera; Phoca (hispida, vitulina, largha, grypus) and Pagophilus (groenlandica)



Chapter 3

Population Structure and Genetic Diversity of Antarctic Pack Ice seals in Relation to Life History Traits

Introduction

Six species of pinnipeds breed south of the Antarctic Convergence, located between 50 and 60°S, the natural boundary of the Antarctic region. Two species, the southern elephant seal (*Mirounga leonina*) and the Antarctic fur seal (*Arctocephalus forsteri*) breed mainly on subantarctic islands near the Antarctic Convergence and to the north of it, and their pelagic distribution exists to the north of the Antarctic pack ice (Laws, 1981). In contrast, the four species of true seals (Phocidae; *Lobodon carcinophagus*, *Hydrurga leptonyx*, *Ommatophoca rossii*, *Leptonychotes weddellii*) of the monophyletic tribe Lobodontini are largely confined to the pack ice regions and fast ice of the Antarctica.

Although the Antarctic pack ice seals (crabeater, leopard, Ross and Weddell seals), share a circumpolar distribution, they are ecologically partitioned due to differences in life history traits (Siniff, 1991). Ecological partitioning results from differences in utilization of, and preference for, different sea-ice habitats, in response to the need for a stable ice platform for parturition and mating, and access to the marine environment for foraging (Bartholomew, 1970; Stirling, 1983). Although much thought has been focused on the evolution of mating systems in relation to breeding habitat and other factors (Stirling, 1983; Cassini, 1999), it is unclear how availability, access to, and stability of habitat for parturition influences genetic diversity and population structure within species. In most pinniped species, parturition is temporally synchronized, and the distribution of individuals during the breeding season is likely to strongly influence differentiation of populations. However, it is also possible that population structure is affected by associations of related individuals outside of the breeding season. Therefore, fidelity to specific areas for breeding, dispersal between breeding areas, and mating structure are likely to influence the development of within-species diversity and population structure. However, other life history characteristics, including prey

preference, foraging behaviour, and seasonal migrations are also likely to influence diversity and differentiation.

Descriptions of life history traits for each of the four species of Antarctic pack ice seals have been documented from a large number of previous studies (reviewed in Laws, 1981). Although these descriptions allow for predictions of the type and degree of population structure likely to characterize each species, measuring the actual amount of population structure by conventional methods is challenging. Molecular techniques may be of particular value in studying population structure and diversity where direct observation, either using mark recapture or telemetry, is difficult. The Antarctic pack ice seals may be more amenable to molecular studies because direct observation of movement and dispersal is not feasible for large numbers of individuals, due to the relative inaccessibility of their habitat. The use of molecular markers to study the Antarctic seals may therefore facilitate understanding of the relationship between ecology and population genetics, and provide insight into the relative importance of life history traits in the development of population structure within closely related but ecologically partitioned species.

Ross seals (*Ommatophoca rossii*) are the least known of the four species of Antarctic pack ice seals. Until recently they were only rarely observed around the edge of the consolidated pack ice surrounding Antarctica, likely due to the difficulty of gaining access to this habitat. Population size is estimated at 220,000 individuals (Gilbert and Erickson, 1977). The Ross seal is solitary and has a circumpolar distribution, although it is generally thought to be more abundant in certain areas (Siniff, 1991). Large breeding aggregations have not been observed, leading to the assumption that the mating system of Ross seals is either monogamous or similar to the serial monogamy that characterizes hooded (*Cystophora cristata*) and crabeater (*Lobodon carcinophagus*) seals (Stirling, 1983). Observations of scaring around the neck of adult male Ross seals (personal observation), likely inflicted by conspecific females, would support the contention of a serially monogamous mating system. Breeding has not been observed but is thought to occur in November (Siniff *et al.*, 1970) on drifting ice floes, where a single pup is weaned within one month (Ørtisland, 1970a). Recently, it has been observed that Ross seals undergo a catastrophic moult similar to that of the elephant seals. The moult occurs

in January, when seals haul out for periods of at least several days, and likely longer, on large stable floes. During this time individuals are reluctant to enter the water to forage, as evidenced by the presence of urine and feces on the ice near moulting seals. Therefore, stable ice platforms are important to Ross seals during the breeding season and while moulting. However, recent evidence from satellite telemetry studies suggests that, for the bulk of the annual cycle, individuals are largely pelagic and spend most of their time foraging north of the Antarctic pack ice. Telemetry from more than ten seals outfitted with satellite transmitters has shown that during the Austral winter Ross seals undertake prolonged trips north to feed pelagically in open water, returning to the ice edge only rarely to haul out and rest (Blix and Nordoy, 1998). During this time seals likely feed on cephalopods and some fish.

Three aspects of Ross seal life history suggest that only limited population structure should be found in this species. First, solitary individuals are found throughout the dense pack ice during breeding and moulting, and no large aggregations of animals are observed at any time of the year. Second, the use of drifting ice floes for parturition and mating while distributed at low density suggests that the mating system is likely monogamous or serially monogamous, and the geographic distribution of breeding individuals is not strongly influenced by limitations of access to, or availability of, suitable habitat. And lastly, it is possible that animals returning from foraging excursions north of the pack ice may not return in subsequent years to the same general areas for breeding.

Leopard seals (*Hydrurga leptonyx*) are the largest of the Antarctic pack ice seals, and along with the killer whale (*Orcinus orca*) are the top predators in the Antarctic marine ecosystem. Population estimates vary, but there are assumed to be approximately 220,000 individuals (Gilbert and Erickson, 1977). Generally, solitary individuals are found within the margins of the Antarctic pack ice, although during the later summer months some animals are found deeper in the pack foraging on weaned crabeater seal pups (Siniff and Bengston, 1977). During winter, when there may be increased competition for krill as a food source, large numbers of individuals move north to subantarctic islands, where they hunt at fur seal and penguin colonies. These northward migrations seem to occur at regular 4-5 year intervals, with oscillations of numbers from

few to several hundred seals visiting these hunting grounds (Rounsevell and Eberhard, 1980). In general juvenile leopard seals seem to disperse more extensively and move north in the winter months. Some individuals are known to have returned to hunt at the same Antarctic and subantarctic islands in subsequent years (Walker *et al.*, 1998; Hiruki *et al.*, 1999). Leopard seals are assumed to be foraging generalists, with krill being the dominant food item in the winter, but they are also known to feed on a diverse range of prey including fish, cephalopods, sea birds, and seals (fur and crabeater). Breeding occurs on the outer fringes of the pack ice in December and January, following the pupping season in October to mid November (Ørtisland, 1970a). Only mother – pup groups are found on ice floes and during the breeding season individuals are solitary and sparsely distributed.

Slip *et al.* (1994) suggested that animals found hauled out at subantarctic islands might be recruited from a 'catchment' south of these islands. Therefore, if well-defined subpopulations of leopard seals exist during the mating season, then evidence of this mating structure might be found between islands following the breeding season. Alternatively, if the effect of breeding at low density in the pack ice leads to high levels of genetic exchange between regions, no correlation of geography to genetic differentiation would be expected. Likewise, samples obtained from individuals in pack ice regions following the breeding season may show genetic differentiation, if mating between geographic regions is not random.

Weddell seals (*Leptonychotes weddellii*), have been the most studied of the Antarctic phocids due to their association with the land-fast ice of the Antarctic Continent. Large aggregations of seals found near research stations in McMurdo Sound (McMurdo and Scott Bases), the Vestfold Hills (Davis Base), and on Signy Island (Signy Base), have facilitated extensive research on this species. Populations near these research stations have been the subject of extensive long-term studies examining demography and mating structure in Weddell seals (Stirling, 1969; Siniff *et al.*, 1977; Croxall and Hiby, 1983; Green *et al.*, 1995). Weddell seals display high levels of philopatry in which individuals return annually to breed and have pups in the same areas of land-fast ice. Breeding colonies are found too far from the seasonal ice edge to permit access to open water during the late winter and spring, forcing seals to maintain holes in the ice. Cracks

created by tidal action, glacial movements, and ice sheet dynamics, form in predictable areas and are used by parturient females during pupping. Adult males are attracted by the presence of estrous females and aggressively compete for aquatic territories beneath breathing holes. Mating occurs in the water under the fast ice and cannot be directly observed. However, molecular studies examining male reproductive success have demonstrated that the mating system is moderately polygynous (Gelatt, 2001).

Juvenile and non-breeding individuals are excluded from the breeding colonies until late in the breeding season (Stirling, 1969) and until they reach maturity when they return to their natal areas to breed. Juveniles and subadults tend to aggregate in large groups near the ice edge. Recently however, there have also been observations of large groups of immature seals on large floes in the pack ice. Satellite telemetry suggests that immature seals may spend most of their time in the pack ice (Stewart, 2003) on large drifting ice floes (Boveng, 2001).

Small, relatively isolated Weddell Seal breeding colonies occur at Signy Island, South Orkney Islands, and at Larsen Harbour on the southern end of South Georgia Island. The colony at Larsen Harbour is thought to be the most northerly breeding colony of Weddell seals. Long distances and expanses of unsuitable Weddell Seal habitat isolate these small breeding aggregates. At least one other isolated population of Weddell seals exists at White Island, McMurdo Sound (Stirling, 1972). This population of approximately fifteen breeding adults was recently founded from a small number of individuals but is now physically isolated from other breeding colonies in McMurdo Sound by 20 km of ice sheet (Gelatt, 2001).

Given the high rate of natal philopatry, and moderately polygynous mating system, Weddell seals are expected to show differentiation between breeding colonies and reduced genetic variation within breeding aggregates due to genetic drift and non-random mating. Some gene flow between breeding areas may be expected due to the extensive mixing of immature individuals in the pack ice, and subsequent recruitment to breeding colonies other than their natal site. Immature seals sampled in the pack ice may be mixtures of individuals from different breeding colonies and may be less differentiated than adults sampled during the breeding season. Likewise, breeding areas sampled following the mating season may be less homogenous, as unrelated juveniles may be

permitted into breeding areas following mating (Stirling, 1969). Geographically and physically isolated populations would be expected to show dramatic genetic differentiation, and greatly reduced genetic variation, due to small population size and extensive genetic drift.

Crabeater seals (*Lobodon carcinophagus*) are the most abundant pinniped species, numbering in excess of five million individuals (although some population estimates are even greater; Gilbert and Erickson, 1977). They have a circumpolar distribution in the pack ice but show some preference for the continental shelf slope, where concentrations of krill, their major food source, may exist.

During the October breeding season, crabeater seals form triads on drifting ice floes, consisting of a female, her pup, and an attendant male. The male guards the female from other males until she weans her pup and becomes receptive to mating (Siniff *et al.*, 1979). Under this mating system, termed serial monogamy, males are unlikely to mate with more than a few females.

Juvenile survival may be very low (≈ 20%) and the highest known source of mortality is predation by leopard seals. A large proportion of crabeater seals older then one year have large raking scars from previous leopard seal attacks, suggesting that predator avoidance may be important for weaned crabeater seal pups. Avoidance of leopard seals may be achieved by movements of large numbers of weaned pups into fast ice regions (Hofman, 1975).

Outside of the breeding season, adults are semigregarious, forming small groups ranging from 2-6 individuals (Hofman, 1975), although larger groups have been observed. Satellite telemetry of 19 crabeater seals suggests that individuals move throughout the pack ice region, with young individuals moving more than adults, although one adult has been recorded traveling more than one third the distance around Antarctica in less than eight months (J. L. Bengtson personal communication).

The use of drifting ice floes for mating, a serially monogamous mating system, and large individual movements suggests that crabeater seals may have little genetic structure and that, under a single large panmictic breeding population, genetic diversity should be very high.

In a preliminary study, Davis *et al.*, (2000) used microsatellite genotyping to investigate population structure among three Weddell Seal populations, and individual crabeater seals from the Amundsen and Bellingshausen Seas. Diversity was also measured in a single population of leopard seals. This work demonstrated the ability of microsatellite markers to determine population structure and genetic diversity in three species of Antarctic seals. While the results of their study supported some of the hypotheses about the development of population structure explored above, here I present a more comprehensive study, including multiple populations of each of the four species of Antarctic pack ice seals. By investigating levels of population differentiation and diversity in each species using the same genetic markers, the relative importance of different life history traits to the development of population structure in closely related but ecologically partitioned species can be investigated. We also expect that an understanding of genetic diversity and population structure in Antarctic seal species will be invaluable when considering conservation and management issues, especially when considering the potential consequences of climate change and increased human activity in Antarctica.

Materials and methods

Sample collection

Our goal was to obtain material suitable for DNA extraction from the complete circumpolar distribution of each of the four Antarctic seal species. Samples of fresh skin, muscle tissue, blood, bone, or teeth were obtained during scientific research expeditions, and from animals shot during exploratory sealing expeditions (Ørtisland, 1970b) or for sled dog food near Antarctic research stations. Whenever possible, samples collected at different points in time were used in order to assess the temporal stability of population structure. Thus, while some samples were collected during the 1960s, most were collected from 1980 to the present.

Ninety Ross seal samples were obtained from four geographic regions consisting of the Ross Sea, the Queen Maud Land coast near the Norwegian Base Maudheim, the

Queen Maud Land pack ice between South Africa and their Antarctic Expedition Base SANAE III, and the pack ice surrounding the South Orkney Islands. Samples were obtained as skin, blood, or teeth (Table 3-1a). The geographic location of sampling was not known for the South African sample, but was assumed to be north of the South African base in the pack ice off the Queen Maud Land coast (Figure 3-1a). In order to assess both geographic and temporal variability in population structure each of the four sampled areas were analyzed separately with the two Queen Maud Land samples considered to be temporal replicates separated by approximately ten years.

A total of 148 leopard seal samples were obtained from six geographic regions including one that was sampled in two consecutive years. Samples were obtained from three subantarctic islands, Heard, Macquarie, and Bird Island, South Georgia (sampled in both 1995 and 1996). In addition, samples were collected in pack ice surrounding the South Orkney Islands, along the west side of the Antarctic Peninsula, and in the Eastern Ross Sea - Amundsen Sea (Table 3-1b). Samples were grouped by geographic region of sampling (Figure 3-1b) and samples collected in 1995 and 1996 from Bird Island were analyzed separately to assess changes in population structure over time.

Weddell seals were sampled from breeding colonies near research stations, within the breeding season, and following the breakup of the breeding colonies. Additionally, samples were collected from groups of non-breeding individuals in the pack ice surrounding Antarctica. A total of 893 samples were obtained from 13 colonies sampled during the breeding season, 4 post-breeding aggregates, and 6 groups of non-breeding seals found on large floes in the pack ice surrounding Antarctica (Table 3-1c). Breeding colonies were sampled near the Antarctic research stations McMurdo (7 colonies – including the physically isolated population at White Island), Davis (3 colonies), Syowa (1 colony), Signy (1 colony), and from Larsen Harbour, South Georgia (1 colony). Post-breeding groups were sampled from 4 locations including Syowa base, McMurdo Sound, the Bay of Whales, and Siniff Bay. Non-breeding groups were sampled in four locations in the dense pack ice of the Ross Sea and two groups were sampled off the coast of Queen Maud Land (Figure 3-1c). Samples obtained in the same geographic area, but during different times of the annual breeding cycle, were used to test for differences in population structure during and after breeding. Similarly, samples obtained in the same

geographic region but in different years were used to estimate the temporal stability of genetic structure.

Three hundred and three crabeater seals were sampled as either single individuals or small groups in the pack ice during research expeditions aboard icebreakers (Table 3-1d). The majority of samples were obtained from the eastern Ross Sea, Amundsen Sea, Bellingshausen Sea, and the west coast of the Antarctic Peninsula, although small numbers were also sampled in McMurdo Sound, off the coast of Queen Maud Land and in the Southern Indian Ocean (Figure 3-1d). As there were no breaks in the sampling distribution, and given that only individuals or small groups (maximum N=19) were sampled at any given location, individuals were not subdivided into discrete groups for analysis.

Laboratory analysis

DNA was extracted using the QIAquick DNA extraction system (QIAGEN) from skin, muscle, blood, and bone and tooth drillings. Microsatellite analysis was performed using Applied Biosystems' fluorescence-based technology on either a 373A or 377 automated DNA sequencers. The microsatellite primer sets used for analysis were cloned from three species of Antarctic seals (Davis *et al.*, 2002). In addition one marker cloned from North American Black Bear (G1A) was used (Paetkau and Strobeck, 1994). Different subsets of loci were used in each species, and were selected based on their relative ease of typing in each species as well as their amplification strength with low concentration DNA (from bone and teeth). A total of 15 loci were used in Weddell Seal, 14 in leopard seal, and 9 in each of the Ross Seal and crabeater seal (Table 3-2). Overall, five loci were used in all species. PCR conditions were identical to those used in a preliminary study investigating the population genetic structure of Antarctic seals (Davis *et al.*, 2000).

Statistical analysis

Tests of disequilibrium and genetic diversity

The presence of individuals with matching genotypes was examined using the Microsoft Excel Microsatellite toolkit (Park, 2001). Homogeneity of allele frequency distributions was tested across all pairs of sampled regions using the probability test (Raymond and Rousset, 1995), or where possible exact tests (Guo and Thompson, 1992). Tests were combined across loci (Fisher, 1970). Each pair of loci was tested for linkage disequilibrium within each sampled region using probability tests or exact tests. Deviation from Hardy Weinberg equilibrium (HW), with the alternative hypothesis of heterozygote deficiency, was tested for each locus in each population, in addition to global tests over both loci and populations. All tests of disequilibrium were performed using Genepop on the Web (http://wbiomed.curtin.edu.au/genepop/).

Allelic diversity (number of observed alleles), unbiased expected heterozygosity (H_E; Nei and Roychoudhury, 1974) and observed heterozygosity were calculated for each geographic region. Unbiased estimates of expected heterozygosity and alleleic diversity were also calculated for each species assuming no population subdivision and using the five loci used in common to all species.

Genetic Differentiation

For the three species where sampling design allowed for the placement of individuals into discrete groups (Ross, leopard, and Weddell seals) we used conventional estimates of population genetic structure as follows. Pair-wise estimates of D_S (Nei, 1972) were calculated from allele frequency data in the Gendist application of PHYLIP Version 3.572 (Felsenstein, 1995). Population gene trees were constructed using the Fitch-Margoliash algorithm (Fitch and Margoliash, 1967) and were visualized using the Drawtree application of PHYLIP. Tree branches were rotated to aid in visual presentation and branch lengths adjusted to ensure the scale of trees for each species was consistent. Nonparametric bootstrap replicates (Felsenstein, 1985) were generated using

Freqboot and a majority rule consensus tree of replicate Fitch-Margoliash trees was used to infer support for the observed tree topology. Genepop on the web was used to calculate Weir and Cockerham's (1984) estimate of F_{ST}. Significance of pair-wise F_{ST} values was assessed using a randomization procedure in Arlequin version 2.000 (Schneider *et al.*, 1999).

A genotype assignment test (Paetkau *et al.*, 1995) was used to assign individuals to the population from which their genotype is most likely to occur. The number of cross assignments between geographic regions is indicative of the amount of gene flow between populations, and in cases of strong population structure can be used to identify individual migrants. Genotype assignments were calculated using the program available at http://www2.biology.ualberta.ca/jbrzusto/Doh.php#RunTest. Allele frequencies were adjusted using the method of Titterington *et al.* (1981). Assignment was also used to generate D_{LR}, a genetic distance between populations based on the relative likelihoods of individual genotypes in each pair-wise comparison. D_{LR} has been shown to perform well in empirical studies of genetic distance relative to geographic distance (Paetkau *et al.*, 1997). Randomization tests were used to assess the significance of the assignments of individuals to their nominal (home) population. Under the null hypothesis of a single panmictic population in HWE, 1000 replicate datasets were generated by creating new HW individuals from the total gene pool (with replacement). These new individuals were then partitioned in groups of identical size to the original samples and assignment was calculated. Under this randomization scheme assignment of an individual to its nominal population is expected to be random and can be used to assess the significance of 'home' assignments from the empirical data. Using a type one error of 5%, and 1000 replicates, an excess of 50 randomized datasets that produce equal or more 'home' assignments than the empirical data suggests that these populations are not distinct. However, if greater proportions of 'home' assignments are rare in the randomized datasets (i.e. < 50) there may be evidence population structure.

Weddell Seal samples were classified into four discrete types based on whether they were sampled in the 1) the breeding season; 2) in areas where breeding was known to occur but sampled post breeding; 3) from areas in the pack ice where predominantly non-breeding individuals were observed, or; 4) from isolated colonies (either due to

physical isolation or as indicated by genetic analysis). The relationship between sample type and proportion of home assignments was assessed using a Spearman rank correlation.

The distribution of crabeater seal samples was continuous throughout their distribution in the pack ice from the eastern Ross Sea to the western side of the Antarctic Peninsula. In addition, samples from the Southern Indian Ocean and Queen Maud Land coast were limited in size. As such, individuals could not be easily divided into distinct groups large enough to produce accurate estimates of allele frequency, as is required for conventional estimators of population structure. In order to assess genetic subdivision the Queller and Goodnight (1989) estimator of genetic relatedness 'R' was used to determine differences in relatedness within and between groups. As 'R' could not be calculated for single individuals, the analysis was restricted to group sizes of two or more individuals. Distributions of pair-wise relatedness values, both within and between groups, were tested for normality using a Lilliefors test in SPSS 6.1. Differences in mean relatedness within and between groups was tested using a Mann-Whitney U test.

Geographic distances between sampling areas were calculated using an ocean mask in ArcMap 8.1. The use of this mask forced the line connecting pair-wise points to run through marine regions only. Therefore, the distance between points located on opposite sides of the Antarctic continent was calculated as arcs around the landmass. Average sampling location, calculated for groups of Ross, Leopard, and Weddell seals was used to calculate geographic distance (Figure 3-1a-c). The correlation of geographic distance to genetic distance was examined using two-way Mantel tests (Mantel, 1967). For Ross, leopard, and Weddell seals, the genetic distance F_{ST} was used. Relatedness 'R' between groups was tested against geographic distance for crabeater samples. Due to the existence of small isolated populations of Weddell seals, a matrix of binary (0's between continuous populations, and 1's between isolated and continuous, and between isolated populations) was tested against genetic distance. A significant correlation was taken as evidence of a barrier to gene flow (in addition to geographic distance). Separate two-way tests were performed for each Weddell Seal sample 'type' described above, to test for differences in isolation-by-distance between these groups.

Model Based Clustering

An independent assessment of population subdivision was performed using the program STRUCTURE Version 2.0 (Pritchard *et al.*, 2000; http://pritch.bsd.uchicago.edu/). This program uses a model based, Bayesian cluster analysis to infer population subdivision from multilocus genotypes. One attractive aspect of this procedure is that no *a priori* assumptions are made regarding the origins of individuals, as the analysis dictates the clustering of individuals and can therefore reveal cryptic population structure. The admixture model with correlated allele frequencies was used to estimate the probability of the data given predefined numbers of clusters (K). K = 1-10 were tested for each species using 250 000 total generations with a burn-in period of 50 000 generations. The probability of each K was calculated using Bayes theorem, assuming a uniform prior on K.

Results

Microsatellite genotyping

Complete genotypes were obtained for 1393 of 1433 (97.2%) of the samples typed. Less than one percent of data was missing for each species. Of the 40 individuals that had missing data, 34 were not typed at a single locus and six were missing data for two loci. Unscored genotypes were evenly distributed over loci, suggesting that the presence of a high frequency of null alleles (Paetkau and Strobeck, 1995) at any one locus was not the cause of the inability to completely type all individuals. Considerable diversity was found for each species, and within species no two samples had the same genotype. Therefore, the microsatellite loci used in each species contained sufficient variability for individual identification.

Statistical analysis

Tests of disequilibrium and heterogeneity

Ross seals. With four study areas and nine loci, 36 tests for HW heterozygote deficiency were performed. The number of significant tests was no greater than expected due to type I error if the null hypothesis of no homozygote excess, was true. No significant deviations from HW were detected when a strict Bonferroni correction was used to adjust significance levels for multiple tests. Global tests of HW performed across loci within each population were used to check for significant non-random mating within sampled areas (Wahlund effect). No significant results were found, indicating that sampled areas were free of internal genetic structure. Global tests across populations for each locus were performed to detect the presence of loci with high frequencies of null alleles. No significant results were found, suggesting that most alleles were successfully amplified. Pair-wise tests of linkage disequilibrium between each locus in each population revealed 3 significant tests at a type I error rate of 5%, and one at 1%. These results are not significant given the number of test performed, and no tests were significant using a Bonferroni correction for multiple tests. Therefore loci were considered to be independent and were used for the remaining analysis. Of the 6 pairs of Ross Seal populations, only two had significant differences in allele frequency distributions at α = 0.05, and these were not significant when a Bonferroni correction was applied. Results of tests of disequilibrium are summarized in Table 3-3a.

Leopard seals. Eleven of 98 tests for HW heterozygote deficiency (14 loci in 7 populations) were significant at a type I error rate of 0.05, while only 2 were significant at an error rate of 0.01. Using a Bonferroni correction for multiple tests, no results were considered significant. Global tests of loci over populations and populations over loci suggested the absence of loci with high null frequencies, and significant genetic structure within populations, respectively. Independence of loci was confirmed from the results of tests for linkage disequilibrium. Although ten and three tests returned significant results at α = 0.05 and 0.01 respectively, only one test was significant following a Bonferroni correction. Locus Hl-8 and Lc-28 returned a result of 0.0000 in the Bird Island sample

collected in 1996 (B6), however if disequilibrium existed between a pair of loci we should expect to detect it in a number of well sampled study areas. As disequilibrium was detected for only one pair of loci in one population we continued that analysis under the assumption of independence between loci. Of 21 comparisons, nine pairs of sampled regions had significant differences in allele frequencies across all loci at a type I error rate of 5%. Only three significant differences were observed at a 1% level, and only one was considered significant using a Bonferroni correction. Significant differences were detected between the Western Antarctic Peninsula (PN) and Macquarie Island (MQ), but within this comparison the sample size for MQ was small (N=8). Table 3-3b summarizes the results of tests of disequilibrium and heterogeneity in leopard seals.

Weddell seals. 345 tests for HW were performed across 23 sampled regions and 15 loci in Weddell seals. Twenty-two of these tests had significant deficiency of heterozygotes at an error rate of 5%, and four tests returned significant results at 1%. The number of significant results was not larger than expected considering the number of tests performed and no tests were significant using a strict Bonferroni correction. Significant non-random mating within populations was not detected by global tests across loci within each population. Four and one test(s) over loci within populations had significant HW heterozygote deficiency at $\alpha = 0.05$ and $\alpha = 0.01$ respectively, however no tests were significant using a Bonferroni correction. Tests for loci over populations returned one significant result following correction for multiple tests (locus Hl-8). Although this may suggest the presence of null alleles at this locus, heterozygote deficiency at this locus was found for only 5 of 23 populations. Thus, although there is some evidence for heterozygote deficiency at this locus, the adverse effects regarding determination of population structure should be minimal, and therefore this locus was retained for the remaining analysis. In addition, this locus was used to examine parentage of pups born in a population of Weddell seals at Big Razorback Island, where no evidence for null alleles was observed between known mother-offspring pairs (Gelatt, 2001). Significant non-random association of alleles between loci was detected in 112 (5% type I error), and 37 (1% type I error) tests of linkage disequilibrium between loci in each population. The number of significant tests was not greater than that expected given the number performed. Using a strict Bonferroni correction with an initial $\alpha = 0.05$, eleven tests

returned significant results. Ten pairs of loci (one twice) involving fourteen different loci and eight populations returned probabilities of 0.0000. As no pair of loci gave significant results in more than one population, loci were assumed to be independent. In contrast to Ross and leopard seals, pair-wise tests between populations assessing differences in allele frequencies revealed many more significant results than were expected by chance. Of 253 pair-wise tests for differences in allele frequencies between populations 174 were significant at a 5% level, and 150 were significant at 1%. When corrections were made for multiple tests of significance using the Bonferroni procedure, 117 tests were significant. Significant differentiation was found between the geographically isolated populations at Larsen Harbour, Signy Island, and White Island as well as Long Fjord near Davis station in the Australian Antarctic region. These populations are differentiated from each other and to all other populations. In addition, of 36 tests between populations sampled during the breeding season, 13 displayed significant differentiation. Two of 6 tests between breeding colonies sampled after the mating season suggested differentiation, while no allelic differences were found in 15 pair-wise tests between groups of non-breeding seals sampled in the pack ice. The results of the tests of disequilibrium and homogeneity of allele frequencies are given in Table 3-3c.

Crabeater seals. As the sample of crabeater seals was not divided into discrete geographic regions, only nine tests of HW were performed, one for each locus. Two significant results were obtained at a 5% level of significance; however, no tests were significant at 1%, and no tests were significant following a strict Bonferroni correction. Likewise, only three pairs of loci displayed significant linkage disequilibrium at the 5% level, and none of these was significant at the 1% level, or following Bonferroni correction. Therefore, there was assumed to be no evidence for nonrandom mating within the sample of crabeater seals, and loci were deemed to be independent (Table 3-3d).

Genetic Diversity

Ross seals. Considerable variation was observed at each of the nine loci used to genotype Ross seals. Averaged over the four sampled regions, unbiased expected heterozygosity

ranged from a low of 0.33 at locus Lw-7 to a high of 0.95 at locus Lc-18. Likewise allelic diversity ranged from 3.5 alleles (Lw-7) to 19 alleles (Lc-18) (Table 3-4a). Genetic diversity among the sampled geographic regions was similar, with estimates of H_E ranging from 0.69 in the South African Queen Maud Land sample to 0.74 in the Ross Sea. Allelic diversity was more variable and increased with sample size. Mean number of alleles per locus ranged from a low of 7.44 in the South Orkney Islands (N = 12) to a high of 12.00 in the Ross Sea (N = 42). Due to the limited evidence for heterogeneity of allele frequency distributions among sampled regions, all samples were combined to calculate genetic diversity. Over the five loci used in common to all species, expected heterozygosity was 0.76, comparable to estimates within subdivided groups, while allelic diversity increased to 13.20 alleles per locus (Table 3-5a).

Leopard seals. Of the fourteen loci used to type leopard seals, locus Lw-7 had the lowest genetic variation with an unbiased H_E of 0.32 and 3.29 alleles per locus. Locus Lw-10 had the greatest genetic variation with H_E = 0.94 and 13.71 alleles (Table 3-4b). Within each of the sampled regions (including the replicate samples taken at Bird Island in 1995 and 1996) H_E ranged from 0.72 in both the Ross Sea and Antarctic Peninsula to 0.76 at Heard Island. Allelic diversity was greatest in the sample from Bird Island sampled in 1996, displaying an average of 9.93 alleles per locus, and lowest at Macquarie Island with 5.93 alleles per locus. As above, allelic diversity was strongly influenced by sample size (Table 3-5b). Over the five loci used in common to all species, expected heterozygosity was 0.75, comparable to estimates within subdivided groups, while allelic diversity increased to 13.60 alleles per locus (Table 3-5b).

Weddell seals. Over 23 populations, and considering the 15 loci used in Weddell seals, allelic diversity was lowest at locus Hl-20, with 4.68 alleles, and highest at locus Lw-11 (11.12). Due to an even distribution of allele frequencies, locus Lc-6 had the highest H_E at 0.86, while locus Lc-28 displayed the lowest heterozygosity at 0.30 (Table 3-4c). Four Weddell Seal populations had lower expected heterozygosities than the remaining groups. White Island, physically isolated from other Weddell Seal populations by 20 km of ice shelf and known to be founded by a small number of individuals (Gelatt, 2001), displayed the lowest H_E (0.56). Signy Island and Larsen Harbour, isolated by long distances of unsuitable Weddell Seal habitat, also had lower H_E (0.63 and 0.66

respectively). Long Fjord, a deep and narrow fjord near Davis station, displayed low heterozygosity (0.64). The remaining populations sampled from the land-fast ice and pack ice surrounding the Antarctic continent had heterozygosities ranging from 0.72-0.78. Allelic diversity in these groups ranged from 6.07 to 10.87 (Table 3-5c), and was influenced by sample size. As evidence of population structure existed from tests of homogeneity of allele frequency distributions, genetic diversity was calculated by averaging over populations using the 5 loci used to type all species. Across all sampled regions, the average H_E was 0.67 (allelic diversity 7.06). However, when the four isolated populations were removed, heterozygosity increased to 0.69 (allelic diversity 7.91).

Crabeater seals. Estimates of unbiased expected heterozygosity for the nine loci used to genotype crabeater seals ranged from a low of 0.69 for locus Lw-10 to 0.94 for locus Lw-4. Allelic diversity ranged from 10 to 50 observed alleles for loci Lw-10 and Lw-4 respectively (Table 3-4d). The average diversity over the 303 individuals typed was $H_E = 0.85$, allelic diversity 20.67 (Table 3-5d). When only the five loci used to type all species were considered, heterozygosity was 0.82, with 16.60 alleles per locus (Table 3-5d).

Using only the five loci typed in all species, and excluding apparently isolated Weddell Seal colonies, genetic diversity was lowest in Weddell seals. Leopard and Ross seals displayed intermediate, and almost identical, levels of genetic diversity. Crabeater seals had the highest levels of diversity among the four species. Generally diversity was not higher for loci used in the species from which they were cloned. Loci that displayed either high or low diversity in one species generally also did so in all other species. Trends in diversity between species were also apparent at the one locus (G1A) originally cloned from North American black bear (Paetkau and Strobeck, 1994).

Genetic Distance, genotype Assignment, and population Structure

Ross seals. As suggested by the analysis of homogeneity of allele frequency distributions, little evidence for population structure was found in Ross seals. Nei's standard distance (D_S) ranged from 0.0456 between the Ross Sea sample and the

Norwegian Queen Maud Land sample to 0.1142 between the Ross Sea and the South Orkney Islands (data not shown). A phenogram of population relationships suggested a close grouping of the geographically distant Ross Sea population and the Norwegian Queen Maud Land sample, and was found in 68% of bootstrap replicates (Figure 3-2a). Similarly, estimates of pair-wise F_{ST} were small and only two comparisons were significantly different from zero at a 5% level of significance. No significant comparisons remained when a strict Bonferroni correction was made for the six pair-wise tests. Over all populations, F_{ST} was 0.006 (+/- 0.003) and the 95% confidence interval contained zero. D_{LR}, the mean genotype log likelihood ratio across individuals from two populations, indicated low levels of genetic differentiation (Table 3-6a). In two of six cases D_{LR} was negative, suggesting that the genotype of individuals sampled in one population were more likely in the population they were being compared to. The maximum pair-wise D_{LR} value was 2.18 between the South African Queen Maud Land sample and the South Orkney Islands, the two samples with the smallest sample sizes (N = 16 and 12 respectively). On average only 29% of individuals were assigned to their nominal population, and the number of home assignments was significant for only the South African Queen Maud Land sample (Table 3-7a). Overall measures of genetic distance were likely influenced by the small size of the South Orkney Island and South African Queen Maud Land samples. Considering only the Ross Sea and Norwegian Queen Maud land samples, which had the largest sample sizes, no evidence for genetic differentiation was found. Results from the two-way Mantel test suggested no support for increasing genetic differentiation over greater geographic distances (F_{ST} vs. Geographic; r = 0.32951, p = 0.21279).

Leopard seals. Values of D_S varied widely between sampled regions of leopard seals, and were strongly influenced by sample size. The topology of the Fitch-Margoliash tree (Figure 3-2b) showed that poorly sampled populations (Ross Sea, Heard and Macquarie Islands) had increased genetic distance relative to other populations. Topology of the remaining populations was star-like and did not indicate any obvious relationship to geography. Temporal replicate samples taken at Bird Island did not group together in the tree suggesting that individuals sampled in different years are no more genetically related than individuals from different geographic regions. No clusters in the

tree were supported with greater than 50% bootstrap support. Likewise, estimates of pair-wise F_{ST} were small and only one comparison, between the Ross Sea and Heard Island, was significant at a type-one error rate of 5%. No significant pair-wise F_{ST} values were found following a strict Bonferroni correction. Considering all populations overall, F_{ST} was 0.001 +/- 0.002 and was not significantly different from zero. Estimates of differentiation from D_{LR} were similar to other genetic distances, and were largest between samples of small size (Table 3-6b). Only 18% of individuals were assigned to their nominal population and the number of 'home' assignments was significant for only the Antarctic Peninsula sample (Table 3-7b). Genetic distance was not correlated to genetic distance using a two-way Mantel test ($r = 0.11848$, $p = 0.33367$).

Weddell seals. Four populations of Weddell seals displayed marked genetic differentiation from each other and to populations sampled in the land-fast and pack ice surrounding the Antarctic continent. Signy Island and Larsen Harbour grouped together in 100% of bootstrap trees, strongly supporting a genetic relationship between these populations even though the genetic distance between them was relatively large. Weddell seals sampled at White Island, a physically isolated population, were also genetically distinct. Surprisingly, the population of Weddell seals sampled in Long Fjord, near the Australian base Davis, were as genetically distinct as the physically, and geographically isolated populations noted above, even though there is no evidence for isolation of this population. Additionally, the Long Fjord population was differentiated from populations in nearby (< 20 km) Tryne Fjord, and McCallie Rocks (Figure 3-1c). Genetic differentiation among the remaining Weddell Seal populations was limited and there was no obvious relationship of genetic divergence to geographic proximity of sampling locations (Figure 3-2c). Of 253 pair-wise estimates of F_{ST}, 105 were significantly greater than zero following a strict Bonferroni correction. Pair-wise F_{ST} among the four strongly differentiated populations, and between these populations and the remaining continental populations (82 comparisons) ranged from a low of 0.0454 between Larsen Harbour and Signy Island to a high of 0.2118 between Long Fjord and White Island. All were highly significant. Among the remaining 177 pair-wise comparisons there were 23 significant estimates of F_{ST}. Most (19 of 23) were between geographically distant populations sampled during the breeding season, however

differentiation was much lower with F_{ST} ranging from 0.0067 – 0.0213 (Table 3-6c). In contrast to the Long Fjord population in the Australian Antarctic region, populations sampled during the breeding season in close geographic proximity in the fast ice of McMurdo Sound, were not significantly differentiated. Over all populations, F_{ST} was significantly greater than zero (0.03 +/- 0.003). With the exclusion of the four isolated populations F_{ST} remained significant (0.006 +/- 0.001) and suggested evidence for some, limited, genetic structure in Weddell seals.

Estimates of genetic differentiation using D_{LR} (Table 3-6c) were identical to F_{ST} as the two measures were highly correlated (Mantel test; r = 0.98157, p = 0.001). Assignment test randomizations suggested significant levels of 'home' assignment in each of the four strongly differentiated populations as well as in five populations sampled within the breeding season (Table 3-7c). Overall, 25.4% of individuals assigned to their nominal population. However, when the four strongly differentiated populations were not included, this value dropped to less than 10%. When populations were grouped by general geography into eight groups consisting of the Ross Sea (BW, SB, FA, FB, FC, CC), McMurdo Sound (ST, BR, OS, ON, CL, LB, CW), Davis Base (TF, MR), Syowa (S1, S2), Queen Maud Land (Q1, Q2), Northern Islands (SG, LH), White Island (WI), and Long Fjord (LF), 58.3% of individuals were correctly assigned to their 'home' group. As before, when strongly differentiated groups were excluded (SG, LH, WI, and LF) home group assignments were reduced to 36.4%. Therefore, it would seem likely that geographic proximity is an important factor influencing the development of population structure in Weddell seals, at least on a macro-geographic scale. The effect of location of sampling (land-fast ice vs. pack ice) and annual cycle (breeding season vs. post breeding) on the proportion of home assignments was examined by classifying samples into four general types: 1) breeding (land-fast ice); 2) post breeding (land-fast ice); 3) non-breeding (pack ice); and 4) isolated populations. A Spearman rank correlation was used to examine the relationship of sample 'type' to proportion of home assignments. A strongly significant correlation (r = 0.8479, p = <0.0001) suggests that stronger population structure exists during the breeding season than post breeding, and that little population structure exists among aggregations of non-breeding individuals. On average, only 1.5% of non-breeding individuals sampled in the pack ice are assigned to these non-

breeding groups (remaining individuals are assigned to breeding locations) while 11% of post breeding, and 15% of breeding individuals are assigned to their nominal populations. The above results suggest that the development of population structure in Weddell seals is mediated by increasing geographic distance and is more pronounced between breeding colonies, both during and after the breeding season, than it is for non-breeding individuals.

In order to further investigate the effect of geographic distance on genetic differentiation a series of Mantel tests were performed. First, the correlation between pair-wise F_{ST} and geographic distance was examined. Using all 23 populations, genetic and geographic distances were weakly associated ($r = 0.16631$, $p = 0.09191$). A 'barrier' matrix of 0's between continental, 'undifferentiated' populations and 1's both between isolated populations and between these strongly differentiated groups and continental populations was strongly correlated to genetic distance ($r = 0.94114$, $p = 0.001$). Therefore a three-way Mantel test (Smouse *et al.*, 1986) was performed assuming that the effect of this 'barrier' matrix on genetic distance was greater than that of geographic distance, resulting in a highly significant correlation ($r = 0.94049$, $p = 0.001$). Overall, this is strong evidence that, for strongly differentiated populations, geographic distance is not a major factor in their genetic isolation, and that other reasons (barriers to gene flow, genetic drift due to small population size) make more important contributions to the large genetic differences. Therefore, a Mantel test using only the relatively undifferentiated ($N = 19$) populations was performed. Among these populations, genetic distance (F_{ST}) was significantly correlated to geographic distance ($r = 0.85594$, $p = 0.001$). When separate tests were performed for breeding colonies sampled during the breeding season ($N = 9$; $r = 0.85594$, $p = 0.001$), breeding colonies sampled after the mating season ($N = 4$; $r = -0.82562$, $p = 0.22677$), and non-breeding areas ($N = 6$; $r = 0.29321$, $p = 0.22577$) it was apparent that most isolation by distance was caused by the effect of breeding colonies.

Crabeater seals. Assessment of population structure in crabeater seals was performed in a novel manner. Average relatedness, as estimated using Queller and Goodnight's statistic 'R' (1989), within groups of seals sampled in the pack ice was compared to pair-wise relatedness between groups (Figure 3-3). Significantly greater relatedness within groups, as compared to between groups, could be interpreted as

evidence of population structure. Distributions of within and between group relatedness were tested for normality using a Lilliefors test in SPSS 6.1, and the difference in means was tested using a Mann-Whitney U test (Table 3-8). Mean relatedness within groups was 0.011 (σ = 0.074), slightly higher than the value of –0.005 (σ = 0.039) between groups, however the difference in means was not significantly significant (U = 23745.5 W = 31574.5 Z = -1.4435 p = 0.1489).

Pair-wise relatedness between groups was related to geographic distance for a subset of the groups included in the analysis above. In the more densely sampled areas of the Eastern Ross Sea, Amundsen Sea, and Bellingshausen Sea, groups consisting of more than five individuals were used. For the remaining areas any group consisting of at least 2 individuals were included in the analysis. Relatedness 'R' was not correlated to geographic distance using a two-way Mantel test (r = -0.07724 p = 0.16583).

Model based clustering

The Bayesian model based clustering analysis Structure (version 2.0; Pritchard *et al*, 2000) was used to infer population structure without using prior information about the geographic origin of samples. For each species, all typed samples were included in the analysis and were used to estimate the posterior probability ln Pr (X|K) of the data (X) given a specified number of clusters (K). One to ten clusters were tested for each species and the probability of each K (Pr K|X) was calculated from ln Pr (X|K) assuming a uniform prior probability on K. Results were highly congruent with those obtained using traditional methods of population structure analysis. For both crabeater and Ross seals, Pr (K|X) was highest for K = 1, suggesting no population subdivision (Table 3-9). Likelihood estimates of the leopard seal data over one to ten clusters was bimodal and provided support for both K = 1 (0.2497) and K = 6 (0.7503). At K = 6 inferred ancestry in each of the clusters was evenly distributed within both populations and individuals and therefore was relatively uninformative. Therefore K = 1 was accepted as the best explanation of the data, which suggested no population structuring in leopard seals. For Weddell seals, strong support was found for three population clusters (Table 3-9), with an asymmetric number of individuals assigned to each cluster. Most of the inferred ancestry

of individuals sampled at Signy Island and Larsen Harbour was placed in cluster one, whereas the inferred ancestry of Long Fjord individuals was attributed to cluster two. A high proportion of the ancestry of White Island individuals was found in cluster three. Remaining populations had ancestry in both clusters one and three with higher values occurring in the latter. Under the less likely K = 5 a clear distinction was observed between Signy Island – Larsen Harbour, Long Fjord, White Island, and the remaining continental populations (Table 3-10). Therefore, support was found for the genetic separation of strongly differentiated populations, with little evidence for population structuring in weakly differentiated populations.

Discussion

For many species of pinnipeds, individuals can be sampled in large numbers during the breeding season at traditional terrestrial pupping colonies (Stirling, 1983). Assessment of population structure in other southern hemisphere pinnipeds has focused on the differentiation of breeding colonies and has shown high levels of population structure in some species including the southern elephant seal (Hoelzel *et al*, 2001; Slade *et al.*, 1998), New Zealand fur seal (Lento *et al.*, 1994), and subantarctic fur seal (Wynen *et al.*, 2000). Past exploitation and subsequent range expansion in some species, (Wynen *et al.*, 2000) including the Antarctic fur seal, has so diminished the amount of genetic differentiation between breeding colonies, that the detection of pre-exploitation population structure is now impossible. Others species, including the Guadalupe fur seal (Bernardi *et al.*, 1998) and Juan Fernández fur seal (Goldsworthy *et al.*, 2000) have been shown to have higher levels of diversity than expected given their history of exploitation, yet the degree of contemporary and historical population structure for these species is unknown. Under the expectations formed by mating structure theory (Bartholomew, 1970; Stirling, 1983; Cassini, 1999) species that use predictable terrestrial areas for breeding, where females are present at high densities, will show increased levels of sexual dimorphism and polygyny. In addition, as individuals return to these predictable breeding areas with high fidelity (philopatry), large amounts of genetic differentiation between breeding areas should result.

In contrast, the Antarctic pack ice seals, with the exception of the Weddell seal cannot be easily sampled during the breeding season. Ross, crabeater and leopard seals are sparsely distributed throughout the heavy early spring pack ice during breeding. The habitat and distribution of breeding individuals not only makes sampling difficult but also is the basis for proposing a likely lack of population structure in these species. Sampling of individuals in the austral summer becomes more feasible as the ice retreats, concentrating seals within a more penetrable ice pack. In addition, leopard seals can often be sampled during the winter on subantarctic islands. Population structure must therefore be estimated outside of the breeding season for the three pack ice breeding species. Although this may cause differentiation (due to groups of related individuals breeding in geographic proximity) to go unnoticed there is little reason to believe that the geographic distribution of individuals during the breeding season is much different from that in the austral summer three months later. Weddell seals, by contrast, can be sampled during the mating season, due to the proximity of their breeding colonies to Antarctic research stations. Sampling individuals within the mating season, in the same geographic locations but following breeding, and in the Antarctic pack ice 'niche' of younger individuals, allows for investigation of differences in population structure during different times of the season, and at different life stages.

Stirling (1983) suggested that in polar regions, there are generally two types of ice on which seals breed. Polar pack ice offers an unlimited substrate for parturient females, however due to the unstable nature of this habitat low densities of females appear to be favoured by most species. Due to low densities of females, polygyny and sexual dimorphism are less likely to develop. As pack ice is unstable, unpredictable, and abundant, breeding site fidelity does not develop, and population structure is not expected. Land-fast ice, in contract is more limited, although not to the same extent as terrestrial breeding areas. Access to land-fast ice is limited by the need for breathing holes, that form in predictable areas each year. Cracks are limited and therefore, in the absence of terrestrial predators, multiple parturient females must share breathing holes during the pupping season (Stirling, 1977). Moderate polygyny results as males can restrict access to competitors, thus monopolizing breeding opportunities with more than one female. Although seasonal climatic and environmental variation may affect the

quality of individual ice cracks, most form at predictable locations, promoting breeding site fidelity. Individuals, and eventually their offspring, return annually to mate and breed in the same areas of fast ice, resulting the development of population structure.

Inferences of population structure from microsatellites presented in the current study conformed to expectations, based on life history characteristics of the four species of Antarctic pack ice seals and evolutionary theory. No evidence for population structure was found in Ross seals, a pack ice breeding seal that spends much of the year feeding alone in the open water north of the ice pack. High rates of gene flow were indicated among geographically distant populations in the Ross Sea and in the pack ice off Queen Maud Land. Samples collected in the same general area ten years apart were as different as geographically distant populations. Although recent tracking of a limited number of Ross seals using satellite telemetry suggests that individuals return to the same areas of the pack ice in the year following tagging (Blix and Nordoy, 1998), it is possible that over the lifetime of a seal fidelity to a particular region of pack ice is lost. Fidelity to specific sites for breeding and moulting would be difficult to maintain because of the high degree of annual variability in the distribution of ice generally and specific habitat types in the unstable and unpredictable pack ice. The result is genetic homogenization of individuals throughout the range of Ross seals, and a single panmictic population. The closely related Ross seal and southern elephant seal offer an interesting comparison in ecological partitioning. Both species make long foraging trips to the open sea, returning to breeding grounds only to mate and moult. Southern elephant seals return to highly predictable, spatially limited subantarctic islands to the north of the feeding areas to breed, and consequently display marked population structure (Slade *et al.*, 1998; Hoelzel *et al.*, 2001). In contrast Ross seals return to unpredictable, unlimited pack ice for breeding at very low densities, to the south of the feeding areas, and display no population structure.

Leopard seals sampled from six geographic locations in the pack ice and at three subantarctic islands displayed no genetic differentiation. No evidence for population structure was found in individuals sampled in pack ice regions after the breeding season and populations sampled at subantarctic islands were not more related to areas directly to the south in the pack ice (Slip *et al.*, 1994). Although population structure would not be

expected in leopard seals given their breeding habitat, these results contradict two observations that would suggest that some limited population structure might exist. Firstly, geographic variations in leopard seal vocalizations have been found between geographically distant locations at Palmer Peninsula and McMurdo Sound (Thomas and Golladay, 1995). Secondly, as estimated from the resighting of tagged seals, as many as 35 % of individuals return to subantarctic islands to hunt in subsequent years (Walker *et al.*, 1998). The discrepancy between these observations and the lack of population structure indicated by genetic markers may be explained by hunting behaviour. It is likely that related seals, such as mother – offspring pairs, do not hunt together given that greater than 70% of seals found at subantarctic islands in winter are sexually immature (Walker *et al.*, 1998). Seals may return to areas where they have foraging success and become specialists at hunting certain kinds of prey. Hiruki *et al.* (1999) found evidence for cooperative hunting in leopard seals and it is possible that vocal dialects are learned (Abgrall *et al.*, 2003) from hunting partners. Therefore the development of geographically distinct vocal repertoires may due more to social than genetic factors.

The degree of population structure in Weddell seals ranged from virtually absent between groups of non-breeding seals sampled in the pack ice to complete isolation for physically and geographically separated populations. As expected, moderate levels of population structure were observed between breeding colonies, with less differentiation found in breeding areas sampled following the mating season. Previous studies examining protein variation in Weddell seals from different breeding areas around the Antarctic continent found significant differences in allozyme frequencies (Shaughnessy, 1969; Seal *et al.*, 1970; Hofman, 1975). These studies were performed at a large geographic scale and indicate differentiation between breeding colonies over long distances (> 1000 km). Although the populations used in this study were sampled from the same general areas used for previous allozyme studies, we sampled multiple breeding colonies within each region, allowing for an assessment of minimum distances over which population differentiation might occur. Significant genetic differentiation was found at a minimum geographic distance of approximately 700 km (between McMurdo Sound and the Bay of Whales). Differentiation, using analysis of vocal repertoires has been found at similar distances in studies examining differences in underwater

vocalizations at macrogeographic (approximately 5000 km), and mesogeographic (600-2000 km) scales (Thomas and Stirling, 1983; Thomas *et al.*, 1988; Abgrall *et al.*, 2003). No significant population structure was found at smaller distances, suggesting that dispersal over relatively short distances (less than 700 km) results in enough gene flow to effectively homogenize populations. Genetic distance between breeding colonies was significantly correlated to physical distance supporting the contention that recruitment of an individual to a colony, other than place of birth, is a limited by dispersal range. Juvenile dispersal is the most likely method of gene flow. Young Weddell seals probably spend most of their time foraging in the pack ice (Stewart, 2003), where mixing of individuals born at different coastal breeding colonies occurs. When these seals mature a small portion do not return to their natal sites to breed.

The high degree of differentiation and apparent isolation of Weddell seal colonies on islands in the north of the species range, suggests that long distance movements are rare. Isolation of these populations is expected not only because they are isolated by long distances but also because large expanses of unsuitable habitat exist between them and coastal breeding colonies. The small land breeding colony at Larsen Harbour, South Georgia, may be maintained by low levels of immigration from Signy Island (Croxall and Hiby, 1983), as evidenced by the small number of seals sampled at Larsen Harbour assigned to Signy in the genotype assignment test.

Weddell seals can also become physically isolated in the southern extremes of their range. Stirling (1972) identified a small apparently isolated colony of Weddell seals breeding at White Island, separated from nearby colonies in McMurdo Sound, by twenty kilometers of ice shelf. No exchange of tagged seals has ever been observed between White Island and breeding colonies in south-eastern McMurdo Sound, suggesting that the colony is completely isolated. Genetic studies have shown that the colony was founded within the last 100 years by fewer than 10 individuals (Gelatt, 2001). High pup mortality and reduced reproductive rate may indicate inbreeding depression in this population. The founders of this population may have gained access to White Island following a brief break in the McMurdo ice shelf between 1947 and 1956, and were subsequently isolated. In the southern extremes of their range, Weddell seals may also become isolated due to infrequent geological events. At least one other known colony of Weddell seals, isolated

by an ice sheet, exists in the Bunger Hills (Harry Burton, personal communication) suggesting that this mode of isolation may occur periodically. Recent calving of large icebergs, such as B-15 in March of 2000, may also have dramatic effects on Weddell seal breeding colonies. Modification of the pack ice cover by B-15 has been implicated in a reduction of up to 40% in primary production in the Ross Sea (Arrigo *et al.*, 2003), and lower numbers of Weddell Seals in traditional breeding colonies in McMurdo Sound (Cameron, 2001). It is possible that the presence of this barrier to movement could also affect rates of immigration or emigration of Weddell seals from McMurdo Sound. Additionally, the calving of B-15 near the Bay of Whales likely affected the Weddell Seal breeding colonies present there since it removed the protected areas of annual ice cracks in the ice shelf that were used for pupping and mating colonies..

The high degree of differentiation of the breeding colony of Weddell seals in Long Fjord, near Davis station in the Australian Antarctic region, was not anticipated. There is no evidence that this colony was ever physically isolated, and it is unlikely that seals persisted in the Fjord during the last glacial maximum (Hodgson *et al.*, 2001). Even if seals were isolated over geological timescales, seals now freely leave the Fjord and apparently few, if any, overwinter there (Harry Burton, personal communication). Regardless via some mechanism, isolation has been maintained for at least the last five years (1995-2000), as samples of individuals over that time period are strongly differentiated from nearby colonies (approximately 10 km) in Tryne Fjord and at McCallie Rocks. Of 80 seals sampled over this time in Long Fjord, only four individuals possessed genotypes that would suggest that they were immigrants (three from Tryne Fjord and one from McCallie Rocks). Due to the lack of evidence for physical and geographic isolation, it may be reasonable to suggest that seals in Long Fjord have developed behavioural characteristics that allow them to exclude 'unrelated' individuals from immigrating. Behavioural isolation suggests that breeding habitat within the fjord may, in some unknown way, be superior to that of nearby areas. Long Fjord is 25 km long and only 5km wide, and is restricted by a narrow and shallow entrance. The physical characteristics of Long Fjord may accentuate what is a more general pattern of behavioural isolation in Weddell seals. From the data presented here, it would seem that generally speaking, breeding colonies in more southerly regions of land-fast ice display

greater genetic differentiation. These locations may provide a longer lasting, and more stable platform for pupping, and therefore their use may be restricted by individuals who traditionally pup there.

Previous molecular examinations of differentiation in crabeater seals, have failed to find any evidence of population structure, using allozymes (Seal *et al.*, 1970; Hofman, 1975) or RAPD's (Gelatt *et al.*, 1995). Evidence from an analysis of relatedness within and between groups of crabeater seals, presented here, also suggested that a single panmictic population exists in the pack ice surrounding Antarctica. The use of floating pack ice for breeding and large movements by individual seals likely contribute to high levels of gene flow between regions around Antarctica. Weaned crabeater seal pups may form large groups in the land-fast ice in order to avoid predation by leopard seals (Hofman, 1975). If separate breeding aggregates of crabeater seals existed, we may expect to find genetic differences between the groups of young seals before they disperse. As only one sample of pups was collected, this hypothesis could not be tested. However, relatedness within this group was –0.0091, near the average of within group relatedness calculated across all 48 groups sampled. This suggests that even for pups sampled in the pack ice, before they have dispersed great distances from their birth site, relatedness within groups is not large.

Indirect inference of population structure of the four species studied here can be made from estimates of diversity within each species. Population estimates for the Antarctic seals are generally regarded as poor, but relative estimates of abundance may be reasonably accurate (Gilbert and Erickson, 1977). The diversity across the five loci used to genotype all four species correspond to population estimates considering the amount of genetic differentiation observed in each species. Crabeater seals, numbering in excess of five million individuals displayed extremely high levels of genetic diversity. Given this population size and the apparent lack of genetic structure in this species, the effects of genetic drift are expected to be minimal and a large amount of diversity would be predicted. Leopard and Ross seals are thought to be similarly abundant, with overall population estimates around 200,000 individuals. We found no evidence of population structure in either species, and almost identical levels of diversity. Weddell seals, in contrast, had the lowest levels of diversity, even though there are approximately 800,000

individuals. The effect of genetic drift is more pronounced in this species, because of reduced effective population size and to population subdivision. Microsatellite diversity in all four Antarctic species is high when compared to other phocid species including the grey seal (Allen *et al.*, 1995), European harbour seal (Goodman, 1998), southern elephant seal (Hoelzel *et al.*, 2001), but is comparable to the northern ice breeding ringed seal (Palo *et al.*, 2001). Comparative estimates of genetic diversity not only provide indirect estimates of population structure, but may also be important indicators of the genetic health of populations of phocids (Kretzman *et al.*, 2001; Hoelzel *et al.*, 2002).

The effect of pack ice breeding and large individual movements restricts the development of population structure in Ross, leopard and crabeater seals. The Weddell seal, in contrast, displays a wide spectrum of levels of genetic differentiation. Population structure in this species is likely due to high rates of return to predictable breeding areas in the land-fast ice of the Antarctic continent, but may also be influenced by geographic, physical, and behavioural effects.

There is little history of human impact on Antarctic seal populations. Except for the harvest of small numbers of Weddell seals for dog food near Antarctic research stations and two small-scale exploratory sealing expeditions undertaken by Norway (Ørtisland, 1970b), and the former USSR, the Antarctic seals have essentially remained unexploited.. For most pinnipeds species, a history of extensive exploitation may now obscure what the 'natural' level of diversity and population structure may have originally been. Research on seals in the Antarctic allows for assessment of genetic diversity and population structure in an undisturbed environment and provides a benchmark of genetic diversity from which to assess the impact of increasing human activity and environmental change in the Antarctic region.

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Table 3-1. Geographic location, two letter identifier, sample size, date of sampling, and type of sample for a) Ross seals, b) leopard seals, c) Weddell seals, and d) crabeater seals. 'Type' refers to Weddell seal populations that were sampled in nonbreeding (NB), breeding (B), and post breeding (PB) groups.

a) Ross

Population	ID	N	Sample Type	Date
Ross Sea	RS	42	Skin	1999-2000
Queen Maud Land (Norwegian)	NA	20	Blood	2001
Queen Maud Land (South African)	QM	16	Teeth	1979-1988
South Orkney Islands	SO	12	Teeth	1964

b) leopard

Population	ID	N	Sample Type	Date
Ross Sea	RS	13	Skin	1999-2000
Antarctic Penninsula	PN	32	Teeth	1985-1988
South Orkney Islands	SO	33	Teeth	1964
Bird Island, Gouth Georgia	B5	21	Skin	1995
Bird Island, Gouth Georgia	B6	34	Skin	1996
Macquarie Island	MQ	7	Blood	1988
Heard Island	HD	8	Blood	1988

c) Weddell

Population	ID	N	Sample Type	Date	Type
Queen Maud Land 1	Q1	9	Blood	2001	NB
Queen Maud Land 2	Q2	11	Blood	2001	NB
Syowa 1	S1	62	Skin	1999	B
Syowa 2	S2	14	Skin	1999	PB
Tryne Fjord	TF	34	Blood/Skin	1995-2000	B
McCallie Rocks	MR	25	Blood/Skin	1995-2000	B
Long Fjord	LF	80	Blood/Skin	1995-2000	B
Cape Washington	CW	45	Skin	1997-1999	B
Out North	ON	114	Skin	1997-1999	B
Out South	OS	85	Skin	1997-1999	B
Scott Base	ST	30	Bone	1966	B
Big Razorback Island	BR	96	Skin	1996	B
Close	CL	61	Skin	1997-1999	B
Lewis Bay	LB	15	Skin	1997-1999	B
White Island	WI	18	Blood/Skin	1991-2000	B
Bay of Whales	BW	38	Skin	1999-2000	PB
Cape Colbeck	CC	23	Skin	1999-2000	NB
Floe A	FA	32	Skin	1999-2000	NB
Floe B	FB	17	Skin	1999-2000	NB
Floe C	FC	16	Skin	1999-2000	NB
Siniff Bay	SB	29	Skin	1999-2000	PB
Signy Island, South Orkney Islands	SG	26	Skin	1996	B
Larson Harbor, South Georgia	LH	13	Skin	1998	B

d) crabeater

Population	N	Sample Type	Date
Ross Sea	233	Skin	1999-2000
RV Nathanial Palmer	23	Blood	1994
Antarctic Penninsula	29	Muscle	1986
USCGC Polar Star	8	Blood	1995
Aurora Australis	6	Blood	1993
McMurdo Sound	4	Skin	1996-1999

Table 3-2. Microsatellite loci from Davis *et al.* (2002) used to genotype four species of Antarctic pack ice seals.

Locus	Lw-4	Lw-7	Lw-10	Lw-11	Lw-16	Lw-20	Hl-8	Hl-14	Hl-15	Hl-16	Hl-20	Lc-6	Lc-13	Lc-18	Lc-26	Lc-28	G1A	Total
Crabeater Seal	√		√	√			√			√		√			√	√	√	9
Ross Seal		√	√				√		√	√	√			√		√	√	9
Leopard Seal	√	√	√		√	√	√	√	√	√	√		√	√		√	√	14
Weddell Seal	√	√	√	√	√	√	√	√	√	√	√	√			√	√	√	15

Table 3-3. Results of tests of disequilibrium including Hardy-Weinberg equilibrium, linkage disequilibrium, and heterogeneity of allele distributions for: a) Ross seal, b) leopard seal, c) Weddell seal, and d) crabeater seal. Observed number of tests returning significant results and the number expected due to Type I error if null hypothesizes are correct. Two values of significance are show. Bonferroni corrections were made for multiple tests with an initial value of α = 0.05 (adjusted values given in square brackets)

a) Ross

	Individual HW		Global HW (pops)		Global HW (loci)		Linkage disequilibrium		Allele distributions	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
P < 0.05	1	1.80	0	0.20	0	0.45	3	7.20	2	0.30
P < 0.01	1	0.36	0	0.04	0	0.09	1	1.44	0	0.06
Bonferroni	0	[0.00139]	0	[0.01250]	0	[0.00560]	0	[0.00035]	0	[0.00833]

b) leopard

	Individual HW		Global HW (pops)		Global HW (loci)		Linkage disequilibrium		Allele distributions	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
P < 0.05	11	4.90	1	0.35	3	0.70	10	31.85	9	1.05
P < 0.01	2	0.98	0	0.07	0	0.14	3	6.37	2	0.21
Bonferroni	0	[0.00064]	0	[0.00714]	0	[0.00357]	1	[0.00008]	1	[0.00238]

c)Weddell

	Individual HW		Global HW (pops)		Global HW (loci)		Linkage disequilibrium		Allele distributions	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
P < 0.05	22	17.25	4	1.15	2	0.75	112	120.75	174	12.65
P < 0.01	4	3.45	1	0.23	1	0.15	37	24.15	148	2.53
Bonferroni	0	[0.00014]	0	[0.00217]	1	[0.00333]	11	[0.00002]	117	[0.00020]

d) crabeater

	Individual HW		Linkage disequilibrium	
	Observed	Expected	Observed	Expected
P < 0.05	2	0.45	3	1.80
P < 0.01	0	0.09	0	0.36
Bonferroni	0	[0.00560]	0	[0.00139]

Table 3-4. Genetic variation of loci used to genotype: a) Ross seals, b) leopard seals, c) Weddell seals, and d) crabeater seals. Diversity expressed as allelic diversity (A) and expected heterozygosity (H_E). Range of estimates across populations is given in parenthesis. Species source of microsatellite markers is also shown.

a) Ross

Locus	Source Species	A	H_E
Lw-7	Weddell seal	3.50 (3-4)	0.33 (0.23-0.48)
Lw-10	Weddell seal	13.75 (10-19)	0.91 (0.73-0.84)
Hl-8	Leopard seal	9.25 (6-13)	0.81 (0.73-0.84)
Hl-15	Leopard seal	4.00 (3-5)	0.60 (0.55-0.65)
Hl-16	Leopard seal	8.25 (8-9)	0.65 (0.57-0.71)
Hl-20	Leopard seal	10.00 (7-13)	0.82 (0.73-0.88)
Lc-18	Crabeater seal	19.00 (14-27)	0.95 (0.92-0.97)
Lc-28	Crabeater seal	4.25 (4-5)	0.55 (0.48-0.62)
G1A	Black bear	11.50 (8-14)	0.86 (0.84-0.88)

b) leopard

Locus	Source Species	A	H_E
Lw-4	Weddell seal	11.60 (7-17)	0.87 (0.79-0.90)
Lw-7	Weddell seal	3.29 (2-4)	0.32 (0.14-0.46)
Lw-10	Weddell seal	13.71 (10-16)	0.94 (0.90-0.97)
Lw-16	Weddell seal	5.00 (2-7)	0.67 (0.53-0.80)
Lw-20	Weddell seal	5.571 (4-8)	0.56 (0.43-0.65)
Hl-8	Leopard seal	10.00 (7-13)	0.87 (0.81-0.91)
Hl-14	Leopard seal	6.14 (4-9)	0.75 (0.72-0.79)
Hl-15	Leopard seal	8.57 (6-11)	0.82 (0.77-0.85)
Hl-16	Leopard seal	3.57 (2-4)	0.32 (0.13-0.50)
Hl-20	Leopard seal	9.43 (8-11)	0.86 (0.76-0.90)
Lc-13	Crabeater seal	9.57 (7-11)	0.88 (0.83-0.93)
Lc-18	Crabeater seal	8.29 (7-10)	0.78 (0.72-0.89)
Lc-28	Crabeater seal	7.57 (6-13)	0.78 (0.72-0.86)
G1A	Black bear	9.30 (5-13)	0.86 (0.82-0.88)

c) Weddell

Locus	Source Species	A	H_E
Lw-4	Weddell seal	9.52 (3-14)	0.80 (0.48-0.92)
Lw-7	Weddell seal	5.90 (3-8)	0.65 (0.45-0.82)
Lw-10	Weddell seal	9.39 (5-14)	0.85 (0.74-0.90)
Lw-11	Weddell seal	11.12 (4-18)	0.83 (0.49-0.90)
Lw-16	Weddell seal	6.08 (4-8)	0.76 (0.60-0.85)
Lw-20	Weddell seal	7.34 (3-12)	0.75 (0.48-0.82)
Hl-8	Leopard seal	9.39 (5-14)	0.85 (0.66-0.91)
Hl-14	Leopard seal	8.51 (4-13)	0.80 (0.69-0.88)
Hl-15	Leopard seal	9.81 (3-20)	0.60 (0.37-0.86)
Hl-16	Leopard seal	5.55 (4-8)	0.63 (0.30-0.74)
Hl-20	Leopard seal	4.68 (3-6)	0.63 (0.34-0.72)
Lc-6	Crabeater seal	9.86 (6-13)	0.86 (0.56-0.92)
Lc-26	Crabeater seal	8.38 (3-12)	0.80 (0.57-0.87)
Lc-28	Crabeater seal	3.06 (2-5)	0.30 (0.07-0.48)
G1A	Black bear	7.90 (3-11)	0.73 (0.43-0.86)

d) crabeater

Locus	Source Species	A	H_E
Lw-4	Weddell seal	50	0.94
Lw-10	Weddell seal	12	0.69
Lw-11	Weddell seal	20	0.91
Hl-8	Leopard seal	24	0.91
Hl-16	Leopard seal	16	0.89
Lc-6	Crabeater seal	19	0.90
Lc-26	Crabeater seal	14	0.81
Lc-28	Crabeater seal	10	0.68
G1A	Black bear	21	0.92

Table 3-5. Genetic diversity across populations of: a) Ross seals, b) leopard seals, c) Weddell seals, and d) crabeater seals. Diversity expressed as allelic diversity (A), expected heterozygosity (H_E), and observed heterozygosity (H_O). Diversity over the five loci used in all species is also shown.

a) Ross

Population	ID	N	A	H_E	H_O
Ross Sea	RS	42	12.00	0.74	0.75
Norwegian Antarctic Research Expedition	NA	20	9.67	0.73	0.75
Queen Maud Land (South African)	QM	16	8.00	0.69	0.64
South Orkney Islands	SO	12	7.44	0.72	0.72
Total (Mean for A, H_E, H_O)	4	90	9.28	0.72	0.72
Five Loci (one panmictic population)			13.20	0.76	

b) leopard

Population	ID	N	A	H_E	H_O
Ross Sea	RS	13	7.07	0.72	0.70
Antarctic Penninsula	PN	32	8.71	0.72	0.73
South Orkney Islands	SO	33	9.50	0.73	0.69
Bird Island, Gouth Georgia (1995)	B5	21	8.29	0.74	0.74
Bird Island, Gouth Georgia (1996)	B6	34	9.93	0.73	0.74
Macquarie Island	MQ	7	5.93	0.73	0.72
Heard Island	HD	8	6.50	0.76	0.79
Total (Mean for A, H_E, H_O)	7	148	8.24	0.73	0.72
Five Loci (one panmictic population)			13.60	0.75	

c) Weddell

Population	ID	N	A	H_E	H_O
Queen Maud Land 1	Q1	9	6.07	0.73	0.74
Queen Maud Land 2	Q2	11	6.40	0.74	0.75
Syowa 1	S1	62	10.87	0.73	0.73
Syowa 2	S2	14	7.47	0.75	0.71
Tryne Fjord	TF	34	8.67	0.73	0.73
McCallie Rocks	MR	25	8.87	0.75	0.76
Long Fjord	LF	80	6.60	0.64	0.64
Cape Washington	CW	45	9.27	0.75	0.72
Out North	ON	114	10.73	0.74	0.75
Out South	OS	85	10.20	0.75	0.74
Scott Base	ST	30	9.00	0.75	0.74
Big Razorback Island	BR	96	10.53	0.74	0.74
Close	CL	61	9.53	0.74	0.71
Lewis Bay	LB	15	7.53	0.78	0.73
White Island	WI	18	3.93	0.56	0.65
Bay of Whales	BW	38	8.40	0.72	0.72
Cape Colbeck	CC	23	8.40	0.74	0.75
Floe A	FA	32	9.47	0.74	0.74
Floe B	FB	17	8.20	0.78	0.78
Floe C	FC	16	7.53	0.74	0.73
Siniff Bay	SB	29	8.53	0.75	0.72
Signy Island, South Orkney Islands	SG	26	6.07	0.63	0.67
Larson Harbor, South Georgia	LH	13	4.60	0.66	0.68
Total (Mean for A, H_E, H_O)	23	893	8.39	0.73	0.73
Five loci (mean over 23 populations)		23	7.06	0.67	
Five loci (mean over 19 populations)		19	7.91	0.69	

d) crabeater

Population	ID	N	A	HE	HO
Crabeater	All	303	20.67	0.85	0.84
Five Loci (one panmictic population)			16.60	0.82	

Table 3-6. Genetic distance between study populations of: a) Ross seals, b) leopard seals, and c) Weddell seals. F_{ST} (× 100) lower matrix, D_{LR} upper matrix. F_{ST} estimates that are significantly greater than zero are shown in bold.

a) Ross

	RS	NA	QM	SO
RS		-0.45	0.64	1.45
NA	-0.07		0.86	-0.31
QM	1.05	0.56		2.18
SO	1.57	-0.36	0.09	

b) leopard

	RS	PN	SO	B5	B6	MQ	HD
RS		0.96	0.35	0.30	0.40	2.58	1.75
PN	0.45		0.97	-0.01	0.83	3.08	0.86
SO	0.09	0.41		-0.30	-0.02	2.28	-0.14
B5	0.05	-0.41	-0.60		0.20	1.81	-1.36
B6	0.27	0.22	-0.02	0.12		0.86	0.08
MQ	1.10	1.04	0.98	0.62	-0.07		-0.24
HD	2.08	0.47	-0.29	-0.83	0.33	-0.01	

c) Weddell

	Q1	Q2	S1	S2	TF	MR	LF	CW	ON	OS	ST	BR
Q1		-0.79	-0.12	0.46	1.02	0.68	8.74	1.68	1.88	1.61	1.89	1.67
Q2	-0.77		0.56	0.03	1.02	0.69	5.99	0.57	1.07	0.98	0.97	0.76
S1	0.19	1.09		-0.67	1.29	0.53	8.50	1.35	1.16	1.13	1.12	1.26
S2	-0.29	-0.47	-0.11		1.58	0.65	9.53	1.03	0.63	0.80	0.37	0.41
TF	0.95	1.21	0.78	1.17		0.10	4.67	1.82	2.20	1.70	1.62	1.99
MR	0.73	0.83	0.66	0.67	0.12		5.69	0.79	0.65	0.47	0.71	0.96
LF	**8.70**	**8.17**	**7.73**	**9.00**	**5.44**	**5.65**		7.75	8.55	8.29	7.37	7.85
CW	0.57	0.11	**1.29**	0.34	**1.09**	0.57	**7.37**		0.14	0.10	0.01	0.30
ON	0.84	0.79	**1.28**	0.32	**1.54**	0.45	**7.35**	0.07		0.04	-0.14	0.10
OS	0.63	0.78	**1.24**	0.31	**1.15**	0.48	**7.43**	-0.01	-0.01		-0.12	0.05
ST	1.34	1.04	**1.06**	-0.08	**1.31**	0.16	**7.02**	0.37	0.11	0.27		-0.23
BR	0.73	0.36	**1.06**	0.06	**1.12**	0.43	**7.10**	0.08	-0.02	-0.02	0.16	
CL	0.61	0.68	**1.21**	0.21	**1.32**	0.51	**6.77**	0.03	-0.07	0.07	-0.12	0.02
LB	1.24	1.28	**2.03**	0.23	1.28	0.76	**7.03**	0.07	0.61	-0.02	0.29	0.38
WI	**14.59**	**9.43**	**11.36**	**11.05**	**13.44**	**12.13**	**16.67**	**10.20**	**10.52**	**10.66**	**10.95**	**10.05**
BW	1.56	1.23	**1.78**	0.46	**1.56**	0.78	**8.17**	0.38	**0.67**	0.75	0.75	0.61
CC	0.33	0.79	1.10	0.21	**1.36**	0.39	**6.71**	0.01	0.15	0.38	-0.01	0.04
FA	0.22	0.73	**0.96**	0.38	**1.69**	0.91	**9.03**	0.27	0.08	0.39	0.71	0.16
FB	1.35	1.47	1.27	-0.28	1.44	1.21	**8.87**	0.46	0.95	1.06	0.57	0.56
FC	-0.55	0.20	0.73	-0.32	0.84	0.35	**8.01**	-0.29	-0.33	-0.30	0.31	-0.28
SB	0.93	0.06	**1.46**	0.40	**2.13**	1.15	**9.01**	0.56	0.51	0.87	1.01	0.64
SG	**11.15**	**10.25**	**9.46**	**9.87**	**10.70**	**9.16**	**15.13**	**9.35**	**8.74**	**9.74**	**8.41**	**9.06**
LH	**12.03**	**9.96**	**10.05**	**10.19**	**10.06**	**8.71**	**13.26**	**9.96**	**9.69**	**9.74**	**8.60**	**9.35**

c) continued

	CL	LB	WI	BW	CC	FA	FB	FC	SB	SG	LH
Q1	1.47	1.27	12.04	2.08	1.17	0.44	1.98	0.20	1.48	10.17	13.48
Q2	0.90	1.22	7.36	2.10	1.08	0.98	1.37	0.21	0.96	6.46	9.22
S1	1.24	1.11	9.97	2.08	1.33	0.21	0.32	0.50	1.60	7.98	11.34
S2	0.67	0.45	10.26	1.17	1.06	0.35	0.17	0.86	1.69	8.38	11.54
TF	1.76	1.24	12.06	2.36	2.33	1.75	1.43	1.33	2.33	8.61	11.21
MR	0.75	0.40	11.02	1.16	1.10	0.40	0.93	0.54	1.58	7.51	9.98
LF	7.72	6.82	15.80	8.44	7.30	9.32	8.45	8.02	8.77	12.31	15.15
CW	0.36	0.02	9.07	0.54	0.46	0.18	0.14	0.14	0.99	7.12	11.35
ON	0.06	0.28	10.34	0.70	0.49	-0.12	0.00	-0.18	0.88	7.82	10.78
OS	0.12	0.03	10.59	0.91	0.48	-0.05	0.17	0.05	1.06	8.03	10.52
ST	-0.33	-0.52	9.90	0.82	0.22	0.05	-0.26	0.18	1.06	7.05	9.41
BR	0.09	0.08	10.18	1.03	0.25	-0.16	-0.23	-0.12	0.83	7.62	10.60
CL		-0.23	10.04	0.90	0.30	-0.02	0.16	-0.22	1.08	7.50	10.24
LB	0.15		11.81	1.79	0.96	0.30	-0.03	0.58	2.03	7.52	9.57
WI	**10.07**	**12.18**		9.21	10.15	10.41	11.44	9.46	10.96	16.30	21.17
BW	0.53	**1.90**	**10.46**		1.03	1.10	1.08	0.32	2.03	9.15	12.35
CC	-0.07	1.15	**11.91**	0.76		0.28	-0.05	0.33	0.67	7.22	11.34
FA	0.33	1.31	**11.70**	**1.15**	0.42		-0.47	-0.42	0.43	7.41	10.38
FB	0.82	0.38	**13.51**	1.25	0.57	0.89		-0.66	0.71	7.25	11.62
FC	-0.36	0.06	**11.31**	0.44	-0.10	-0.46	0.33		0.31	7.20	10.21
SB	0.82	1.63	**12.11**	1.32	0.55	0.52	1.25	0.13		7.35	10.92
SG	**8.84**	**10.37**	**20.79**	**11.27**	**9.50**	**8.69**	**9.01**	**9.69**	**8.68**		4.03
LH	**9.36**	**8.87**	**21.18**	**11.42**	**10.25**	**9.75**	**9.63**	**9.96**	**9.75**	**4.54**	

Table 3-7. Results of the assignment test for: a) Ross seals, b) leopard seals, and c) Weddell seals. Rows contain individuals sampled in each population, while columns indicate the populations to which individuals were assigned. Values in bold indicate groups where significant numbers of individuals assigned to their nominal population.

a) Ross

	RS	NA	QM	SO	Total
RS	20	15	5	2	42
NA	11	3	3	3	20
QM	8	2	**6**	0	16
SO	3	6	1	2	12

b) leopard

	RS	PN	SO	B5	B6	MQ	HD	Total
RS	0	5	4	1	2	0	1	13
PN	0	**12**	6	10	4	0	0	32
SO	1	5	10	4	11	1	1	33
B5	3	5	5	3	4	1	0	21
B6	1	8	9	3	11	1	1	34
MQ	0	1	0	0	4	1	1	7
HD	0	0	3	3	2	0	0	8

c) Weddell

	Q1	Q2	S1	S2	TF	MR	LF	CW	ON	OS	ST	BR
Q1	0	1	4	1	0	0	0	1	0	0	0	0
Q2	1	0	0	1	0	0	0	2	2	2	0	0
S1	2	0	**15**	4	4	3	0	1	3	2	4	2
S2	0	0	6	0	1	0	0	1	1	1	1	1
TF	1	0	4	0	**10**	7	2	1	1	1	0	2
MR	0	0	5	0	2	3	1	1	3	2	0	0
LF	0	0	0	0	3	1	**76**	0	0	0	0	0
CW	0	0	1	1	0	2	0	5	6	5	2	6
ON	1	2	2	3	3	4	0	10	13	15	8	8
OS	0	0	1	0	1	2	0	10	14	**14**	6	6
ST	0	0	2	1	1	2	0	3	4	4	0	2
BR	0	1	2	0	0	2	0	7	10	7	5	**15**
CL	1	1	4	1	2	1	0	6	10	8	4	6
LB	0	0	0	0	0	1	0	2	1	0	4	3
WI	0	0	1	0	0	0	0	0	0	0	0	0
BW	0	0	0	0	0	1	0	4	9	1	4	1
CC	1	0	1	0	0	0	0	4	3	1	1	2
FA	1	0	2	2	0	1	0	1	2	5	2	5
FB	0	0	2	0	0	0	0	2	1	1	0	4
FC	0	0	1	0	0	1	0	0	0	1	1	3
SB	0	0	3	0	1	2	0	3	3	1	3	1
SG	0	0	0	0	0	0	0	0	0	0	0	0
LH	0	0	0	0	0	0	0	0	0	0	0	0

c) continued

	CL	LB	WI	BW	CC	FA	FB	FC	SB	SG	LH	Total
Q1	2	0	0	0	0	0	0	0	0	0	0	9
Q2	1	1	0	0	0	0	0	0	1	0	0	11
S1	2	1	1	1	3	9	1	3	1	0	0	62
S2	0	0	0	1	0	0	1	0	0	0	0	14
TF	1	1	0	0	0	0	2	0	1	0	0	34
MR	2	0	0	0	1	0	1	3	1	0	0	25
LF	0	0	0	0	0	0	0	0	0	0	0	80
CW	2	1	0	4	1	2	3	1	3	0	0	45
ON	13	1	0	8	4	5	0	8	6	0	0	114
OS	11	1	0	2	7	2	3	0	5	0	0	85
ST	6	1	0	0	1	0	1	1	1	0	0	30
BR	8	3	0	10	8	6	3	5	4	0	0	96
CL	4	1	0	1	4	2	0	3	2	0	0	61
LB	3	1	0	0	0	0	0	0	0	0	0	15
WI	0	0	**17**	0	0	0	0	0	0	0	0	18
BW	1	1	0	**13**	1	1	0	1	0	0	0	38
CC	1	0	0	4	0	3	0	0	2	0	0	23
FA	2	1	0	0	0	1	1	2	4	0	0	32
FB	0	0	0	0	2	2	1	1	1	0	0	17
FC	1	0	0	2	1	1	2	0	2	0	0	16
SB	0	1	0	1	3	3	0	1	3	0	0	29
SG	0	0	0	0	0	0	0	1	1	**22**	2	26
LH	0	0	0	0	0	0	0	0	0	0	**13**	13

Table 3-8. Descriptive and statistical analysis of 'within' and 'between' group relatedness 'R' for crabeater seals. Number of tests is given in parentheses.

	Mean	SD	Lilliefors Test	Test for Mean Differences
Within	0.011	0.074	$W_{(48)} = 0.1385$ $P = 0.0219$	Mann-Whitney U $U = 23745.5$, $W = 31574.5$ $Z = -1.4435$ P (two-tailed) = 0.1489
Between	-0.005	0.039	$W_{(1128)} = 0.0421$ $P = 0.0001$	

Table 3-9. Results of cluster analysis using Structure. The log likelihood [ln Pr (X|K)] of the data given a specified number of clusters (K), and posterior support for given Ks [Pr (K|X)] assuming a uniform prior on K. High probabilities for given Ks are shown in bold

	Ross Seal		Leopard Seal		Weddell Seal		Crabeater Seal	
K	ln Pr (X|K)	Pr (K|X)	ln Pr (X|K)	Pr (K|X)	ln Pr (X|K)	Pr (K|X)	ln Pr (X|K)	Pr (K|X)
1	**-3050.0**	**1.0000**	**-7408.6**	**0.2497**	-47942.8	< 0.0001	**-12440.7**	**1.0000**
2	-3062.0	< 0.0001	-7522.0	< 0.0001	-47499.5	< 0.0001	-12510.0	< 0.0001
3	-3078.4	< 0.0001	-7426.6	< 0.0001	**-46926.7**	**1.0000**	-12558.1	< 0.0001
4	-3080.5	< 0.0001	-8335.7	< 0.0001	-47044.8	< 0.0001	-13235.6	< 0.0001
5	-3112.8	< 0.0001	-7610.5	< 0.0001	-47065.7	< 0.0001	-12462.8	< 0.0001
6	-3101.0	< 0.0001	**-7407.5**	**0.7503**	-47274.6	< 0.0001	-12500.7	< 0.0001
7	-3080.0	< 0.0001	-7524.6	< 0.0001	-48229.9	< 0.0001	-12955.2	< 0.0001
8	-3066.1	< 0.0001	-7760.3	< 0.0001	-48606.0	< 0.0001	-12473.3	< 0.0001
9	-3063.2	< 0.0001	-7576.3	< 0.0001	-48929.1	< 0.0001	-13083.7	< 0.0001
10	-3060.8	< 0.0001	-8428.0	< 0.0001	-50481.9	< 0.0001	-12565.7	< 0.0001

Table 3-10. Proportion of ancestry inferred in each of either 3 or 5 clusters determined from Structure analysis in Weddell seals. For each population the highest inferred ancestry in any cluster is highlighted in grey.

	K = 3 Clusters			K = 5 Clusters			
Population	1	2	3	1	2	3 + 4	5
Q1	0.228	0.033	0.740	0.011	0.026	0.781	0.183
Q2	0.332	0.041	0.626	0.067	0.032	0.524	0.377
S1	0.350	0.036	0.614	0.023	0.031	0.660	0.285
S2	0.317	0.016	0.667	0.021	0.012	0.649	0.317
TF	0.371	0.184	0.446	0.022	0.169	0.669	0.140
MR	0.407	0.088	0.505	0.038	0.079	0.640	0.243
LF	0.023	0.949	0.028	0.017	0.923	0.042	0.018
CW	0.443	0.055	0.502	0.035	0.048	0.607	0.309
ON	0.467	0.028	0.504	0.032	0.024	0.676	0.268
OS	0.439	0.029	0.531	0.030	0.026	0.622	0.322
ST	0.490	0.041	0.469	0.037	0.032	0.629	0.302
BR	0.428	0.032	0.541	0.044	0.028	0.591	0.338
CL	0.494	0.041	0.465	0.027	0.037	0.676	0.260
LB	0.411	0.050	0.539	0.028	0.050	0.669	0.252
WI	0.098	0.031	0.871	0.027	0.016	0.042	0.915
BW	0.379	0.027	0.594	0.022	0.024	0.650	0.304
CC	0.384	0.058	0.558	0.037	0.046	0.583	0.334
FA	0.493	0.015	0.492	0.044	0.013	0.702	0.241
FB	0.524	0.015	0.461	0.026	0.014	0.657	0.303
FC	0.420	0.016	0.563	0.026	0.014	0.761	0.200
SB	0.520	0.033	0.447	0.051	0.026	0.731	0.192
SG	0.937	0.033	0.030	0.921	0.016	0.041	0.022
LH	0.923	0.060	0.017	0.960	0.012	0.019	0.009

Figure 3-1a. Sampling location of Ross seals (N = 4). Two-letter codes refer to the geographic names of the location of sampling (see Table 3-1a).

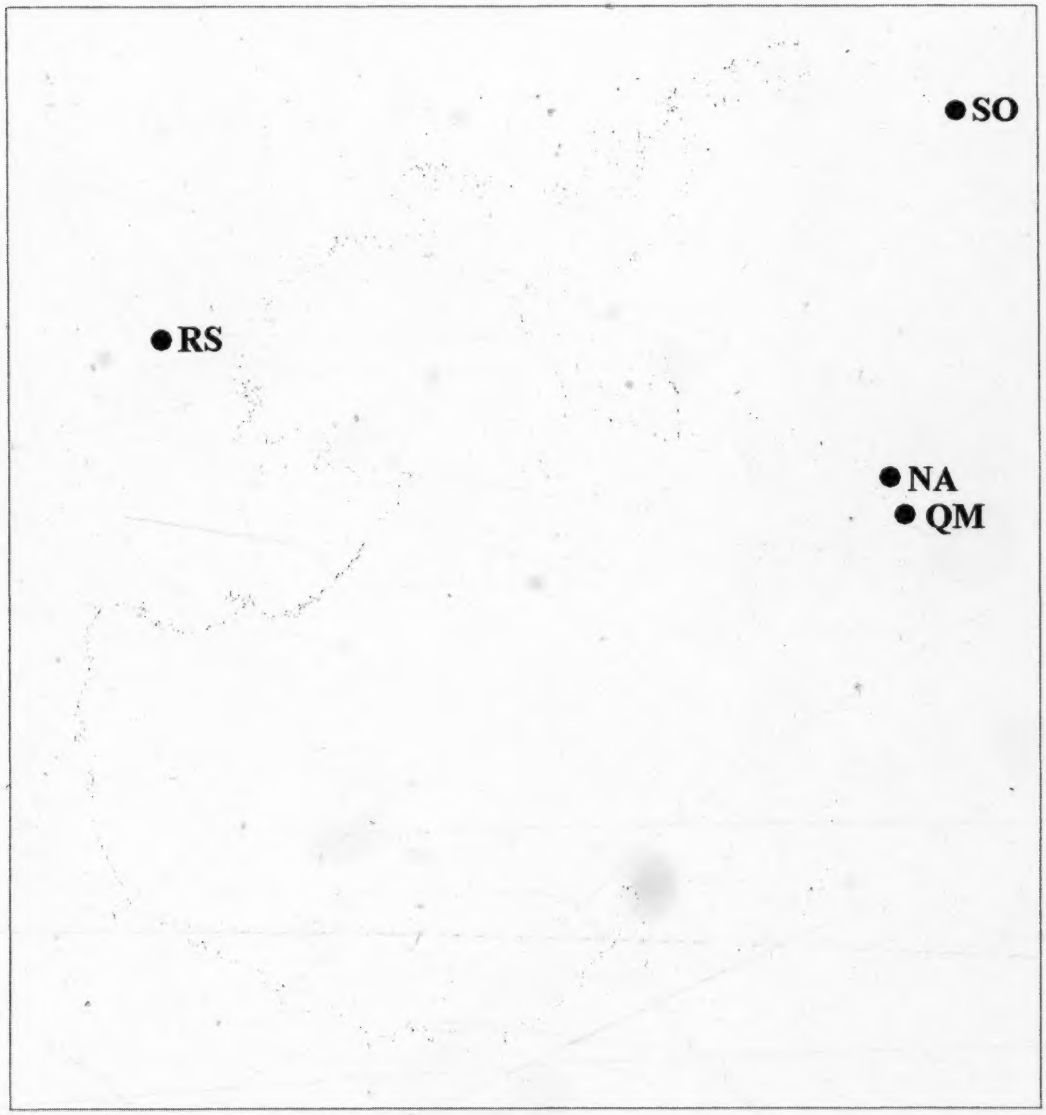


Figure 3-1b. Sampling locations of leopard seals (N = 7). Two-letter codes refer to the geographic names of the location of sampling (see Table 3-1b).

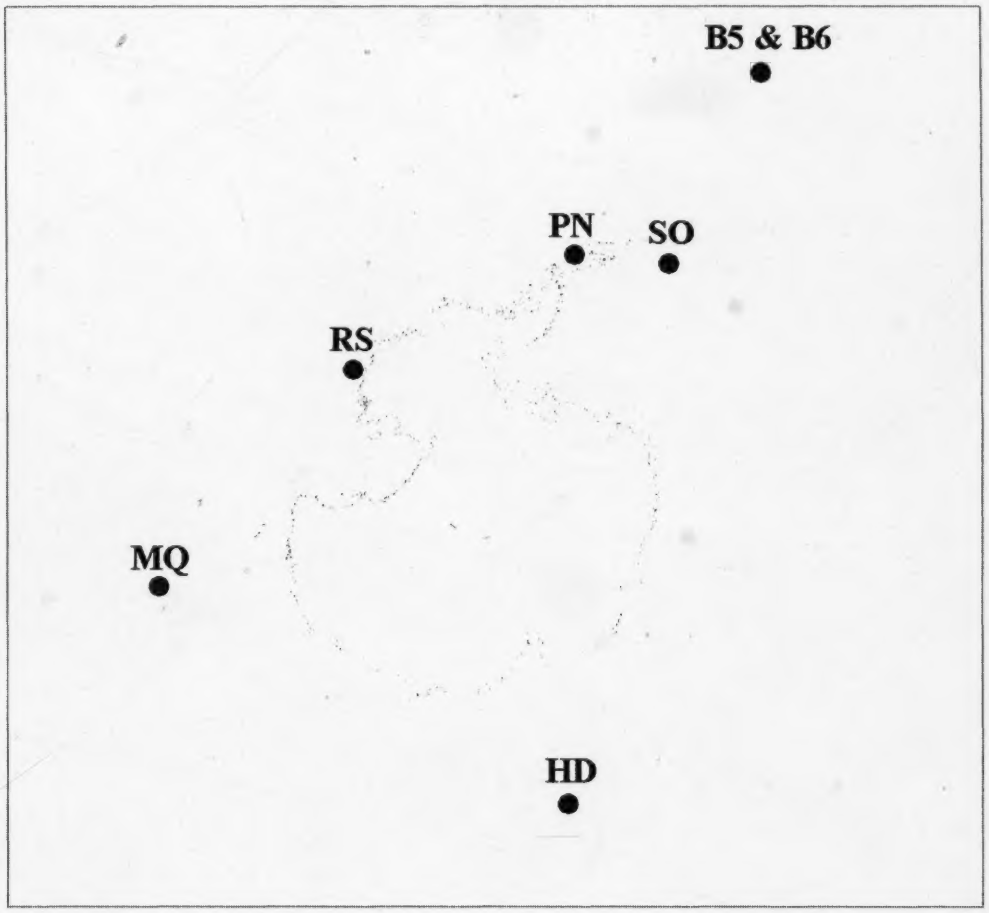


Figure 3-1c. Sampling location of Weddell seals (N = 23). Two-letter codes refer to the geographic names of the location of sampling (see Table 3-1c).

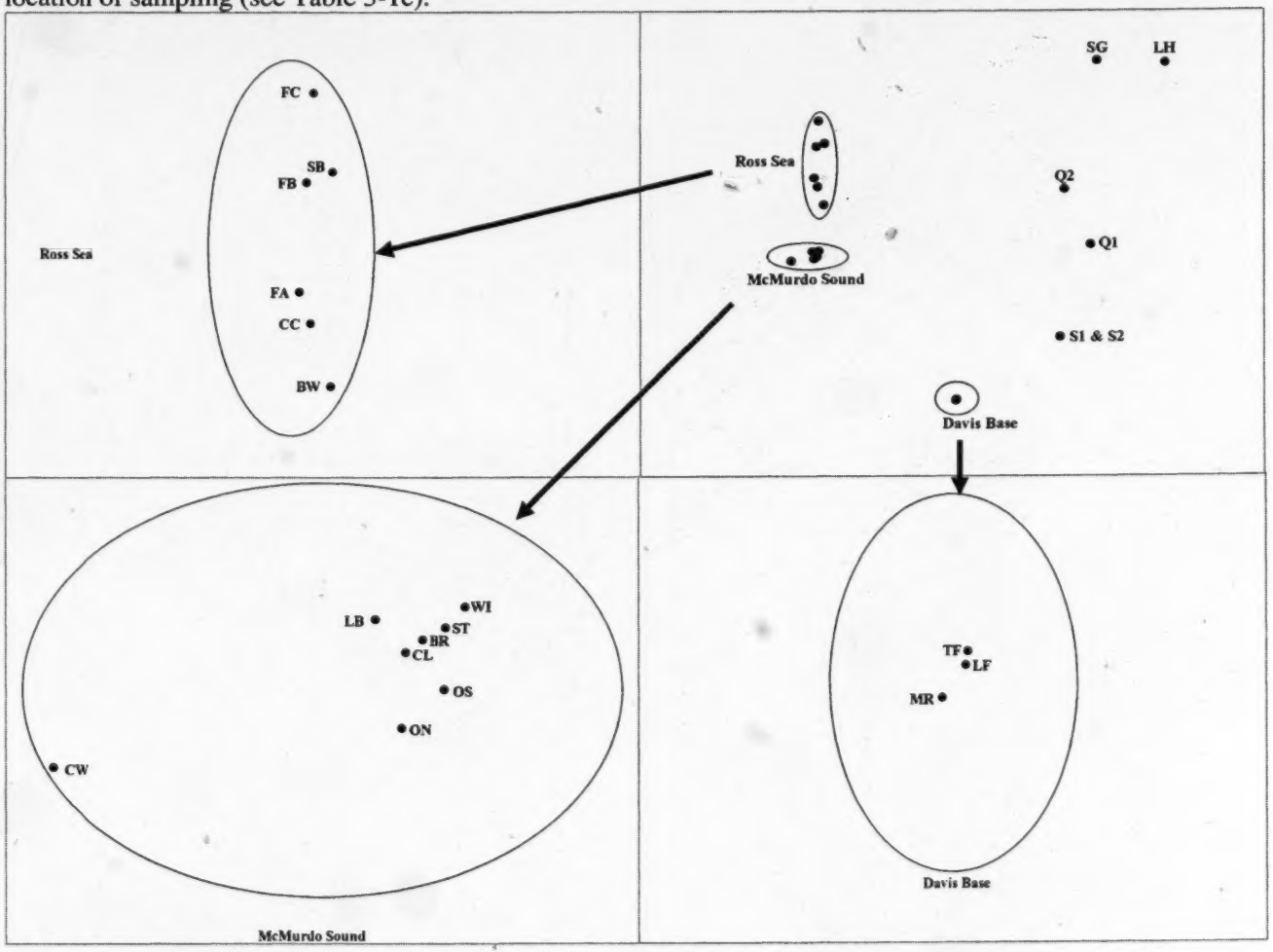


Figure 3-1d. Sampling locations of groups of crabeater seals (N = 48)

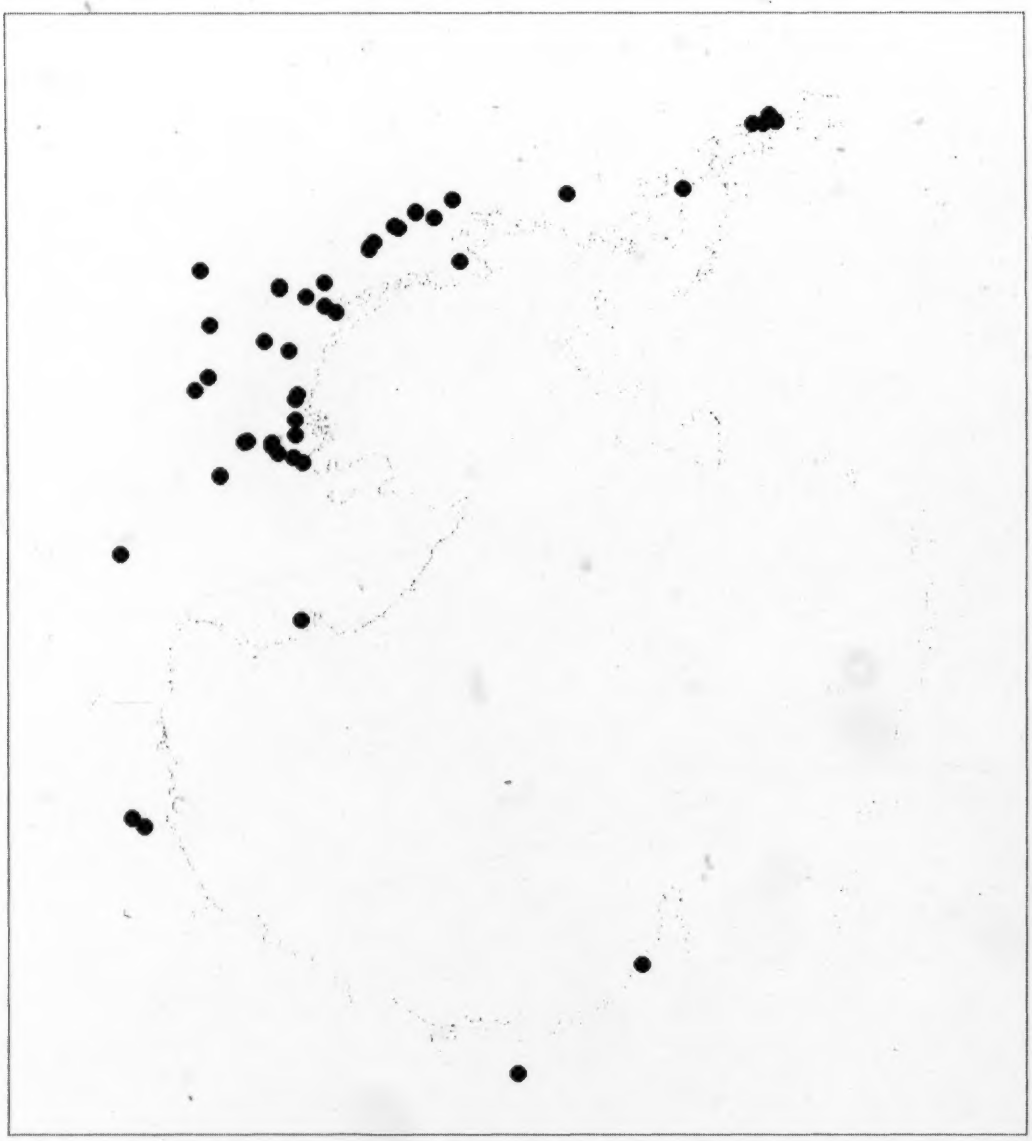


Figure 3-2. Fitch-Margoliash trees of Nei's standard distance (D_S) between study populations for a) Ross seals, b) leopard seals, and c) Weddell Seals. Bootstrap values are shown for clades with greater than 50% support.

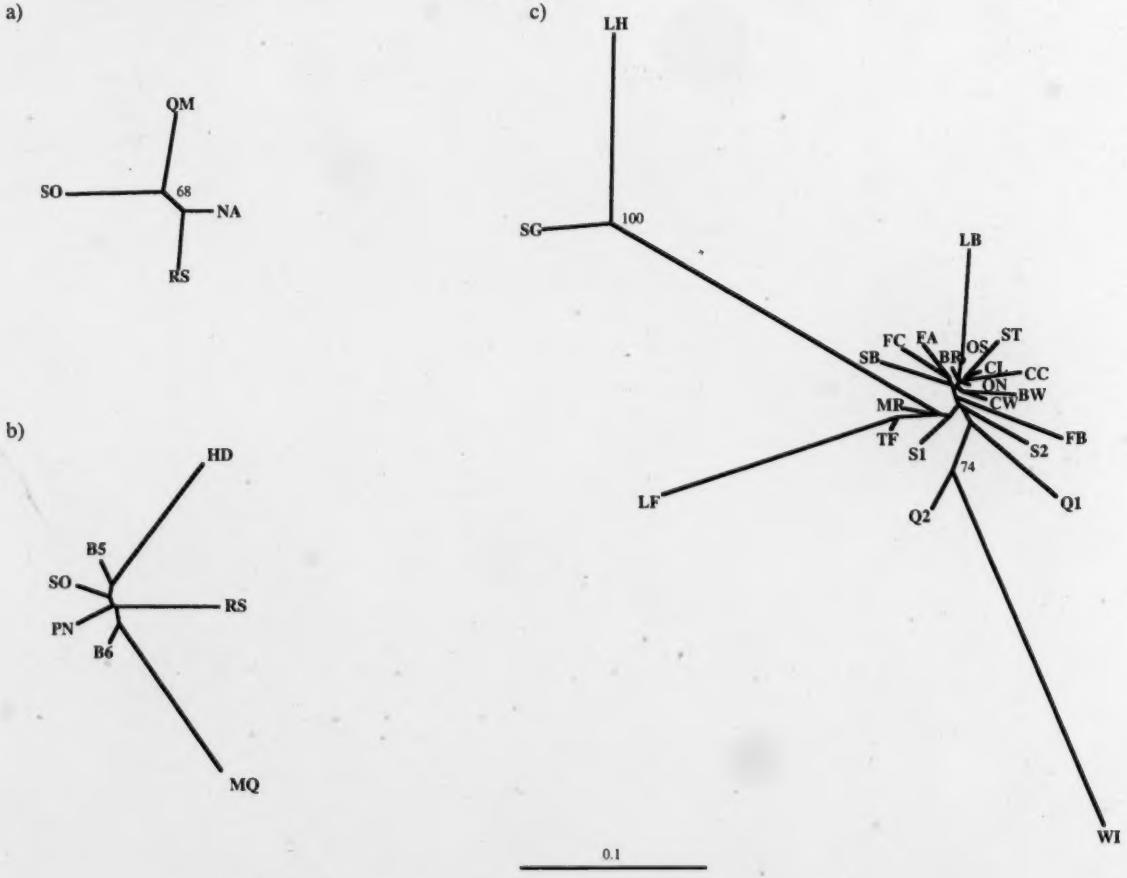
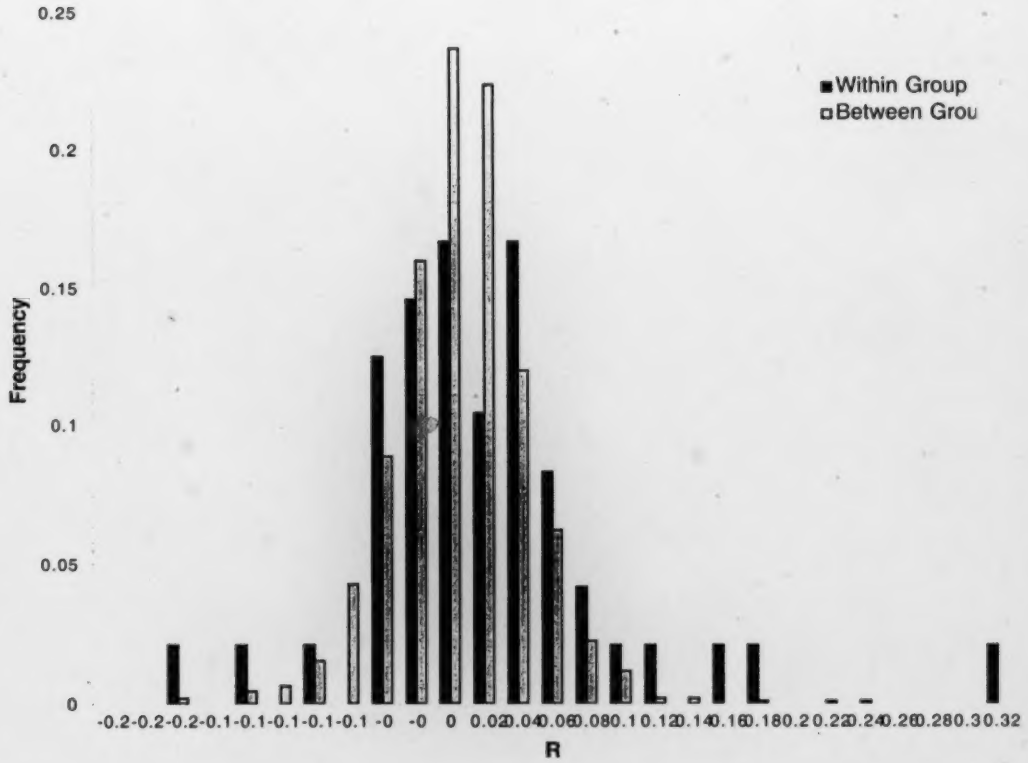


Figure 3-3. Distributions of average relatedness ('R') values within and between groups of crabeater seals.



Chapter 4

Genetic Diversity and Population Structure in Two Arctic Ice Breeding Phocids: Bearded Seals (*Erignathus barbatus*) and Ringed Seals (*Phoca hispida*)

Introduction

Thirteen species of true seal (Phocidae) inhabit the northern hemisphere. Among these, ten species belong to the 'northern' lineage (Phocinae) and inhabit boreal and polar regions, while three belong to the 'southern' lineage (Monachinae) and are found in more temperate and equatorial regions. The use of ice as a substrate for parturition is common among the Phocinae. Six species (bearded, ringed, harp, hooded, spotted and, ribbon seals) give birth to their pups either on land-fast ice or pack ice and mate in the water, while two species (grey and harbour seals) breed largely in terrestrial habitats and mate on land or in the water adjacent to it. Two species (Caspian and Baikal seals) are landlocked endemics closely related to the ringed seal.

Among the species that use ice for breeding, only bearded seals (*Erignathus barbatus*) and ringed seals (*Phoca hispida*) have a circumpolar distribution. Bearded seals are found along the European, Asiatic, and North American coasts of the Arctic Ocean, and on all associated islands, but their northernmost limit occurs between 80 and 85° N. Ringed seals are also found along the coasts of the Arctic Ocean but are found wherever there is open water in fast ice as far north as the pole (King, 1983).

Although their distributions overlap, bearded seals and ringed seals use different sea ice habitats in response to the need for access to the marine environment for feeding while maintaining an association with a stable ice platform for parturition during the breeding season (Bartholomew, 1970; Stirling, 1983). Individuals of both species are solitary throughout most of the year as they forage but come together to interact socially during the brief and yet highly synchronized breeding season. Population structure may develop if related individuals remain in or return annually to the same areas to pup and breed. The predictability and stability of sites for parturition may influence levels of

breeding site fidelity, and hence influence levels of genetic variation within, and differentiation between, breeding groups. In species where individuals return to breed in stable, predictable, geographically distinct areas, genetic differentiation is expected between breeding locations. However when breeding habitat is uniformly distributed over large geographic areas, and/or when individuals move extensively throughout this habitat, little differentiation is expected to result.

The results of previous studies examining habitat preference, and life history of bearded and ringed seals allow for predictions about the amount of population structure likely to characterize each species. Direct observation of individual movements, either by recapture of previously tagged animals or telemetry, over a large portion of their circumpolar range is difficult (Hoelzel, 1997). Therefore molecular methods are used here to evaluate genetic diversity and differentiation in bearded seals and ringed seals from widely spaced locations throughout the Arctic, in hopes of gaining a greater understanding of the relative importance of life history and habitat preference on the development of population structure in these species.

Bearded seals are patchily distributed throughout most of their Arctic and sub-Arctic distribution (Burns, 1981; Kovacs, 2002). Two subspecies of bearded seals have been proposed, and have been named *Erignathus barbatus barbatus* (Erxleben, 1777), occurring from the Laptev Sea west to Hudson Bay, and *E. b. nauticus* (Pallas, 1811), distributed from the Laptev Sea east to the Beaufort Sea. However, the exact distributions of the two forms have not been well defined. Debate also exists over whether the two subspecies actually exist. Two studies examining skull morphology from the same collection have either supported (Manning, 1974) or refuted (Kosygin and Potelov, 1971) the validity of the subspecific designations.

Bearded seals are usually encountered as solitary individuals in their preferred habitat of pack ice over areas of shallow water (McLaren, 1962). They are benthic feeders and for the most part are shallow divers. As a consequence of their preference for shallow water, bearded seals are found mainly in the pack ice or shore leads in coastal areas. They haul out on the edge of moving ice floes that have open leads between them, presumably to reduce polar bear (*Ursus maritimus*) predation (Kingsley and Stirling, 1991) but also to avoid having to self-maintain breathing holes like ringed seals. During

winter, bearded seals are found in areas that contain polynyas, in areas where leads in the ice form in predictable locations, and on the outer edges of pack ice regions. Although they can maintain breathing holes in thin ice, bearded seals tend to remain in areas with open water year round. Some populations are thought to be resident throughout the year, but others may follow the retraction of pack ice northward in the summer, returning southward with the advancing pack ice in winter.

Breeding occurs between late March and mid May, although the exact timing varies geographically (McLaren, 1958a). No aggregations of individuals are observed in the pupping season and females give birth at low density in the open on ice floes. Pups are weaned within 24 days (Lydersen and Kovacs, 1999). Females become receptive to mating shortly after weaning and leaving their pups. Significant evidence suggests that males compete for access to estrous females. Males may fight over access to females (Kovacs, 2002) and also have extensive vocal repertoires (Ray *et al.*, 1969; Van Parijs *et al.*, 2001) that vary both individually (Van Parijs *et al.*, 2003) and between widely separated areas in the arctic (Cleator *et al.*, 1989). Although it has not been demonstrated directly, these characteristics would suggest that the breeding system of bearded seals is moderately polygynous and that separate breeding stocks exist.

Movements of nursing pups have been recorded on a local scale (Hammill *et al.*, 1994), which suggests that mothers and pups generally stay in the same area for long periods of time but can also move short distances (> 10 km) in less than one day. This may be a response to need to move among the small ice floes that are used for nursing because of their instability, but may also be necessary in order to avoid predators. After weaning, most pups move out of the breeding areas and disperse locally along the coast. However, one weaned pup was tracked from Svalbard, west to the Greenland coast, and then south to Jan Mayen (Gjertz *et al.*, 2000). While this represents at least one documented case of long-distance movement in bearded seals, it is not known if such movements result in effective dispersal to distant breeding colonies or whether widely dispersing juveniles eventually return to their natal sites to breed. Generally, the available data on individual movement from satellite telemetry suggest that bearded seals are sedentary and that long-distance movements are rare.

Several aspects of bearded seal ecology and many of their life history traits suggest that significant population structure should characterize the species. Although large individual movements are possible, most individuals are relatively sedentary and remain in areas of pack ice over shallow water. Here they can feed on the ocean bottom and do not need to maintain breathing holes. Although the degree of fidelity to breeding areas is not known directly, most individuals do not move large distances and breeding males have geographically discernable vocal repertoires indicative of discrete breeding areas. Therefore, it is likely that bearded seals will display high levels of differentiation between breeding areas with reduced genetic variation within colonies due to non-random mating and genetic drift.

Ringed seals are the most abundant seal in the northern hemisphere and may number as many as 5-7 million (Stirling and Calvert, 1979). Due to their great abundance and widespread distribution many subspecies have been described. However, the validity of most of these subspecies is probably doubtful, and it is likely that only the landlocked subspecies *Phoca hispida ladogensis* of Lake Ladoga and *P. h. saimensis* of Lake Saimaa are significantly differentiated from the remaining areas where ringed seals (*P. h. hispida*) occur (Amano *et al.*, 2002).

Ringed seals are normally fairly solitary and are found in a diverse array of habitats in the Arctic Ocean. Seals are found in water of virtually any depth, but it is likely that their distribution is driven mainly by food availability and ice conditions (McLaren, 1958b; Lowry *et al.*, 1980). Kingsley *et al.* (1985) found that densities of ringed seals were higher in annual ice than in multi-year ice. Among annual ice types cracking ice was preferred over unbroken ice, floe ice, and rotten ice.

Prime ringed seal breeding habitat has been described as land-fast ice with good snow cover (McLaren, 1958b; Smith and Stirling, 1975) but large stable areas of drifting pack ice have also been shown to have substantial populations of breeding ringed seals (Finley *et al.*, 1983; Wiig *et al.*, 1999). These areas are used by older adults for pupping and breeding, while younger reproductively active individuals are relegated to areas where the ice conditions are less suitable. Immature seals inhabit regions of unconsolidated pack ice, and only come close to the breeding habitat in shear zones between the land-fast ice and the pack ice. Pupping occurs from mid-March to mid-

April, when females give birth to a single pup in a sub nivean lair (Smith and Stirling, 1975). These snow-covered lairs are constructed by hollowing out the snow that accumulates against pressure ridges in the ice, and provide protection from cold, wind, and surface predators. Breathing holes in the ice below the lair are maintained by abrasion of the ice with the claws of the foreflipper.

Breeding occurs in mid-April to mid-May when females ovulate while still lactating. Like Weddell seals in the Antarctic, it seems likely that male ringed seals are able to defend underwater territories by guarding access to the limited number of breathing holes in a fast ice area (Stirling, 1977; 1983). Further evidence of underwater territoriality include that adult males emit a strong odor during the breeding season in spring, have wounds indicative of interspecific fighting, and a large proportion of their underwater vocalizations are antagonistic. These characteristics suggest that males may restrict access to more than one female, resulting in a polygynous mating system.

Among adult and juvenile ringed seals, contrasting patterns of site fidelity and movement exist. Adult seals are assumed to be sedentary (McLaren 1958b) and one male has been resighted in two successive years in the same locality (Smith and Hammill, 1981). In contrast, tagging and telemetry studies performed on juvenile seals have shown that they move large distances (Smith, 1987; Kapel *et al.*, 1998; Teilmann *et al.*, 1999). Although these movements have often been interpreted as migrations, it is unknown whether seals eventually return to breed near their natal site or if they disperse, settle, and breed in distant locations.

It is difficult to predict the amount of population genetic structure that is likely to characterize ringed seals. If adults are sedentary and their offspring migrate but then return to breed in the same area in which they were born, significant population structure may develop. If long-range movements by juveniles result in recruitment to geographically distant breeding areas, genetic differentiation is unlikely. However, the preponderance of long-range movements by such a large portion of tagged juvenile animals suggests the ringed seal populations might be genetically homogeneous.

By investigating levels of population structure and genetic diversity in these two northern ice breeding seals, the relative importance of different life history traits on the development of genetic differentiation can be examined. Factors found to be important in

the development of population structure can be used, along with life history characteristics, to predict the development of structure in other northern ice breeding seals. In addition, an understanding of the degree to which stocks are or are not genetically differentiated is important when considering the potential consequences of factors such as seal harvest and climate change in the Arctic.

Materials and Methods

Sample collection

In order to assess population structure in bearded (*Erignathus barbatus*) and ringed (*Phoca hispida*) seals, specimens were obtained from widely separated areas throughout the geographic range of each species. The sampled areas for both species covered a similar geographic range, encompassing areas from the Bering and Chukchi Seas east through the Canadian Arctic to the White Sea, Russia. Samples were collected as muscle tissue, fresh skin, or bone (skulls), from hunted seals or individuals captured or sacrificed for scientific study. The sampling scheme employed here allowed for an assessment of population structure over large geographic ranges, while also allowing for investigation of differentiation in a limited number of samples collected at smaller distances. Additionally, samples obtained in different years at the same geographic location were used to assess the temporal stability of population structure.

One hundred nineteen bearded seal samples were collected from six geographic areas (Table 4-1a, Figure 4-1a). Specimens were obtained from two regions in close proximity in the Bering Sea (Gulf of Anadyr and St Lawrence Island) during a joint USSR – USA cruise aboard the ZRS Zaslonovo (Hills *et al.*, 1991). Samples from the Eastern Beaufort Sea were collected over almost two decades. Three areas were sampled in the Atlantic Ocean basin including the Newfoundland – Labrador coast (Canada), Qaanaaq (Greenland), and Svalbard (Norway).

A total of 303 ringed seals were sampled from nine geographic locations, including western Alaska, Holman, Minto Inlet, Arviat, Iqualuit, Grise Fjord, Qaanaaq, Svalbard, and the White Sea (Table 4-1b). Seals near Holman were sampled in 1994,

1996, 1998, and 1999 and were used to assess if genetic structure is maintained over time. Most sampling locations were large distances from one another but the Minto Inlet (approximately 100 km north of Holman) sample was included to assess if genetic differentiation can occur over relatively small distances in ringed seals (Figure 4-1b).

Laboratory analysis

DNA was extracted from blood, bone dust, skin or muscle tissue using the QIAquick DNA extraction system (QIAGEN). Microsatellite primer sets were obtained from three sources, including loci cloned from Antarctic seals (Davis *et al.*, 2002), grey seals (Allen *et al.*, 1995; Gemmell *et al.*, 1997) and European harbour seals (Goodman, 1997; Goodman, 1998; Gemmell *et al.*, 1997). A total of fifteen different loci were used (Table 4-2), of which 13 were used to type bearded seal samples and 11 were used in ringed seals. Nine loci were used in common to both species. Microsatellite analysis was performed using an Applied Biosystems 377 automated DNA sequencer. Genotypes were called using Genotyper software (Applied Biosystems) and confirmed manually from gel images. PCR reactions and cycling profiles were used as described in Davis *et al.* (2000).

Statistical analysis

Tests of disequilibrium and heterogeneity

In each sampled area, each locus was tested for Hardy Weinberg (HW) equilibrium using the *U*-test (Raymond and Rousset, 1995) with the specific alternative hypothesis of heterozygote deficiency. Global tests over all loci in each population were used to survey for significant internal structure (nonrandom mating) within sampled areas. Global tests over populations for each locus were used to test for the presence of a high frequency of null alleles (Paetkau and Strobeck, 1995). Within each population each pair of loci was tested for linkage disequilibrium using probability or, whenever possible, exact tests (Guo and Thompson, 1992). Homogeneity of allele frequency distributions between each

sampled region, was assessed using probability or exact tests. Probabilities were combined over the 13 loci used in bearded seals or the 11 loci used in ringed seals (Fisher, 1970). All tests of disequilibrium and heterogeneity were performed using Genepop on the web (http://wbiomed.curtin.edu.au/genepop/).

Genetic variability

Allelic diversity (observed number of alleles) and unbiased expected heterozygosity (Nei and Roychourdhury, 1974) were calculated for each locus in each sampled region. Estimates of allelic diversity and expected heterozygosity were averaged over loci for each sampled geographic region and compared to average observed heterozygosity within regions.

Genetic differentiation

Estimates of D_S (Nei's standard distance; Nei, 1972) were calculated between each pair of sampled regions from allele frequency data in the Genedist application of PHYLIP Version 3.572 (Felsenstein, 1995). Population gene trees were constructed, based on D_S, using the Fitch application of PHYLIP (Fitch and Margoliash, 1967). Bootstrap analysis (1000 replications, Fitch-Margoliash algorithm, majority rule consensus) was used to assess the confidence of the observed tree topology (Felsenstein, 1985).

Genepop on the web was used to calculate Weir and Cockerham's (1984) estimate of F_{ST} over all sampled regions as well as pair-wise between each sampled region. The significance of F_{ST} values were examined using a randomization procedure in Arlequin Version 2.000 (Schneider *et al.*, 1999) to determine if values were significantly greater than zero.

The assignment test (Paetkau *et al.*, 1995; Waser and Strobeck, 1998) was used to assign each individual to the population where their multilocus genotype was most likely to have originated. In general, the number of cross assignments between populations is indicative of the amount of differentiation between populations, and in cases of large amounts of differentiation can be used to identify migrant individuals. Genotype

assignments were calculated simultaneously for all populations using the web based calculator at http://www2.biology.ualberta.ca/jbrzusto.Doh.php#runTest. A randomization test, using 1000 replications, was performed, where new HW individuals were generated from the combined genepool across all populations. These individuals were then subdivided into populations of the same size as the original population and assignment probability was recalculated. In the case of strong population subdivision we would expect that in many of these randomized datasets the number of individuals that assign to their nominal (location of sampling) population would be lower that that observed in our empirical data. Therefore, significant population structure was indicated for populations that had more nominal assignments that would be expected by chance.

The geographic distance between sample locations was calculated using an ocean 'mask' in ArcMap 8.1. This 'mask' required that lines connecting sampling locations (calculated for each pair of samples) run only through water, and not through areas above 85° N for bearded seals (as the distribution of bearded seals does not generally extend to this area). The correlation of geographic distances to genetic distance (F_{ST}) was investigated using two-way Mantel tests (Mantel, 1967).

Model Based Clustering

The program Structure (version 2) was used to independently assess population structure without *a priori* dividing individuals into geographically defined groups (Pritchard *et al.*, 2000). Structure is a model-based clustering method that assigns individuals probabilistically to one of a number of predefined clusters (K). K was treated as unknown and was estimated from the data. The number of clusters indicated by the data was calculated by running the program for K = 1-10, with the admixture model and correlated allele frequencies, for a total of 250 000 generations with a burnin of 50 000 iterations. The probability of each K was calculated using Bayes theorem, assuming a uniform prior probability. The inferred ancestry of individuals sampled in each geographic location, in each of the K clusters most supported by the data, was used to assess geographic population structure.

3. Results

Microsatellite genotyping

Complete genotypes were obtained for all 119 bearded seals typed. No two individuals had the same genotype. Considerable variation was found at the 13 microsatellite markers used, with unbiased estimates of expected heterozygosity ranging from 0.22 for locus SGPV10 to 0.79 at Hl-16, averaged over all populations. Likewise, all 303 ringed seal samples were successfully genotyped at all loci, and no two individuals shared identical multilocus genotypes. Unbiased expected heterozygosity ranged from a low of 0.37 for locus Lw-7 to a high of 0.94 at locus Hl-15. Loci originally cloned from the Antarctic pack ice breeding seals (Davis *et al.*, 2002) were useful in both bearded and ringed seals, and represent an additional source of microsatellite loci for use in phocid species.

Statistical analysis

Tests of disequilibrium and heterogeneity

Bearded Seals. Across 6 populations and using 13 loci, there were a total of 78 tests for HW heterozygote deficiency. Although three tests were significant at a type I error rate of 5%, and one test was significant at 1%, the number of significant tests was not greater than that expected given the number of tests performed. No tests were significant when a strict Bonferroni correction was made for multiple tests of significance. Global tests of HW across populations for each locus resulted in fewer significant results than expected. This result, and the fact that all individuals were successfully typed, indicates that most, if not all, alleles were successfully amplified. Likewise, the number of significant tests performed across loci for each population was not greater than expected due to type I error. Therefore, there was no evidence for genetic structure within any sampled population. Each locus pair was tested for significant non-random association of alleles (linkage disequilibrium) in each population. Twenty tests returned significant results at α

= 0.05, while only two were significant at a 1% error rate. No tests remained significant following a strict Bonferroni correction, indicating independence of loci. Between the six sampled populations there were 15 tests for heterogeneity of allele frequencies. Considerably more tests returned significant results than would be expected due to type I error if the null hypothesis of homogeneity was true. Thirteen pair-wise comparisons were significantly differentiated at both $\alpha = 0.05$ and $\alpha = 0.01$, and all 13 remained significant following a strict Bonferroni correction. The two pairs of study areas for which no significant differentiation was found were Gulf of Anadyr / St. Lawrence Island and Labrador Sea / Qaanaaq. Table 4-3a summarizes the results of the tests of disequilibrium and heterogeneity performed for bearded seals.

Ringed Seals. Among the 132 tests for HW equilibrium performed for each locus (11 loci) in each population (12 samples), 14 returned significant results at a type I error rate of 5%. Five tests were significant at $\alpha = 0.01$ (Table 4-3b). Although slightly more significant tests resulted than would be expected, no tests remained significant following a Bonferroni correction. No single locus or population had a large number of significant results. In order to investigate the possibility that null alleles could be causing the excess of significant tests, global HW tests were performed for each locus over all populations. Two of 11 loci displayed heterozygote deficiency at a type I error of 1%, although neither were significant following a correction for multiple tests. Likewise, one significant test of HW heterozygote deficiency was found ($\alpha = 0.01$) within each sampled area over all loci, although it did not remain significant following a strict Bonferroni correction. Overall the excess of significant tests observed in individual HW tests could not be explained by a high frequency of null alleles at any individual locus or by the presence of significant internal population structure, therefore all loci were used in the remaining analysis. Of 660 pair-wise tests of linkage disequilibrium, for each locus pair in each population, 17 were significant at $\alpha = 0.01$ (33 expected), and 4 were significant at $\alpha = 0.01$ (6.6 expected). Only one test remained significant following a correction for multiple tests. Locus Hl-8 and SGPV11 were in linkage disequilibrium in the sample collected in Holman in 1998. As disequilibrium was for only one pair of loci in one population we continued the analysis under the assumption of independence between loci. Among the 66 pair-wise comparisons between sampled areas, allele frequency

differences were found for 17 populations at a 5% level of significance. Eleven tests returned significant results at $\alpha = 0.01$, and all of these tests remained significant following a Bonferroni correction. Significant allele frequency differences were found between the White Sea and each of the other sampled areas.

Genetic diversity

Bearded Seals. Averaged over the six sampled regions, estimates of unbiased expected heterozygosity ranged from a low of 0.22 at locus SGPV10 to a high of 0.79 at locus Hl-16. Likewise, allelic diversity ranged from 2.33 alleles at both SGPV10 and Hg8.10 to 9.33 alleles at Hl-16 (Table 4-4a). Estimates of expected heterozygosity within each population, over all loci, were lower than observed heterozygosity, except for the Svalbard sample. H_E was relatively constant across sampled areas, ranging from 0.60 in Svalbard to 0.69 in the St. Lawrence Island sample. Allelic diversity was more variable, ranging from 4.00 to 7.54 alleles, and generally increased with increasing sample size. Averaged over all populations and loci, bearded seals were characterized by 5.46 alleles per locus, and an expected heterozygosity of 0.64 (Table 4-5b).

Ringed Seals. Of the eleven loci used to genotype ringed seals, locus Lw-7 had the lowest diversity with 4.67 alleles per locus and an unbiased heterozygosity of 0.37. Locus Hl-15 displayed the highest variation with 19.08 alleles and a H_E of 0.94 (Table 4-4b). Across populations, genetic variation was remarkably constant with expected heterozygosity ranging from 0.78 in the White Sea sample to 0.86 in the sample collected in 1996 at Holman. Allelic diversity was lowest, at 9.55 alleles, in the sample from Holman in 1999 that also had the smallest sample size (N = 11). Excluding this small sample allelic diversity ranged from 11.45 in the White Sea to 14.45 in Iqualuit. Overall ringed seals displayed high levels of genetic variability, characterized by an average unbiased H_E of 0.83 and 12.95 alleles per locus (Table 4-5b).

Genetic distance, genotype assignment, and population structure

Bearded Seals. Three measures of genetic distance were used to estimate genetic divergence between sampled areas. D_S (Nei, 1972), D_{LR} (Paetkau *et al.*, 1995), and F_{ST} (Weir and Cockerham, 1984) suggested similar levels of differentiation. All three genetic distance measures were highly correlated (D_S/F_{ST} r = 0.99 p = 0.002; D_S/D_{LR} r = 0.94 p = 0.002; F_{ST}/D_{LR} r = 0.94 p = 0.002; two way Mantel test). D_S ranged from 0.036 between the neighbouring populations in the Bering Sea (Gulf of Anadyr/St. Lawrence Island) to 0.3161 between the geographically distant St. Lawrence Island and Svalbard samples (data not shown). The topology of the Fitch-Margoliash tree identified three population clusters (Figure 4-2a). The grouping of the Bering Sea populations was supported by strong bootstrap support (100%). The Beaufort Sea sample was highly divergent and grouped with the Bering Sea samples in 64% of bootstrap replicates. Less differentiation was found between regions sampled in the Eastern Canadian Arctic and Svalbard, although a grouping of the Labrador Sea and Svalbard was supported by bootstrap analysis (81%).

Across all sampled areas F_{ST} was 0.064 +/- 0.01, indicating significant population structure. Of the 15 comparisons between sampled areas, estimates of F_{ST} were significant (α = 0.01) for all pairs except Gulf of Anadyr/St. Lawrence Island and Labrador/Qaanaaq (Table 4-5a). This result mimics the results of the tests for homogeneity of allele frequencies, and supports the view of three strongly divergent groups. D_{LR} values, which were highly correlated to F_{ST}, supported the same contention (Table 4-6a).

The assignment test (Paetkau *et al.*, 1995) placed 59% of individuals in the population in which they were sampled. The number of assignments to nominal populations was significant for all six sampled areas (Table 4-7a). Considering the three population clusters that were identified by genetic distances, 92% of individuals were assigned to the cluster from which they were sampled. Cross assignments to another cluster were rare and no individuals sampled in either Bering Sea location were assigned to eastern populations (Labrador Sea, Qaanaaq, Svalbard). Only one individual sampled in the Bering Sea was assigned to the Beaufort Sea, and likewise, one individual sampled

in the Beaufort Sea was assigned to the Bering Sea. Cross assignments between the Beaufort Sea and the Eastern cluster were more common with four individuals sampled in each area being assigned to the other.

F_{ST} was only weakly correlated to geographic distance (r = 0.47127, p = 0.09491) using a two-way Mantel test. The largest values of F_{ST} were found between populations sampled in the Bering Sea and all other populations, suggesting that a barrier to gene flow, in addition to geographic distance, may be contributing to the genetic differentiation observed.

Ringed Seals. The three measures of genetic distance, D_S, D_{LR}, and F_{ST}, were not as strongly correlated in ringed seals (D_S/F_{ST} r = 0.77 p = 0.042; D_S/D_{LR} r = 0.94 p = 0.072; F_{ST}/D_{LR} r = 0.94 p = 0.001; two way Mantel test). Although this may suggest that one or all of these measures are confounded by variance, it may also suggest that in the absence of population structure little correlation should be expected given how much they differ in their calculation. D_S values ranged from 0.0725 to 0.2285 (data not shown). The Fitch-Margoliash tree constructed using these distances was star-like and bootstrap analysis did not strongly support any geographic groupings (Figure 4-2b). In this tree one genetically divergent population, the White Sea, was characterized by its presence on a long branch. Additionally, samples obtained from seals near Holman in 1994, 1996, 1998, and 1999 did not cluster together in the tree.

Over all sampled areas F_{ST} was 0.005 +/- 0.001 supporting the observation of weak differentiation between sampled areas. Of the 66 pair-wise comparisons between sampled areas, only comparisons between the White Sea and each other region had significant F_{ST} estimates (Table 4-6b). Likewise, D_{LR} values between regions were small and often negative. Excluding the White Sea, D_{LR} was never more than one indicating that on average individuals sampled in one region were not more than 10 times more likely to be assigned to their own population than to another. D_{LR} values between the White Sea and each of the other sampled areas in contrast, were 2.77 – 4.26, indicating that on average individuals sampled in the White Sea were 1000 to 10 000 times more likely to be assigned to the White Sea than to any other population.

Only 16% of individuals were assigned to their location of sampling using the assignment test (Table 4-7b). However, 14 of 25 individuals (56%) sampled in the White

Sea were assigned to this population. The number of nominal population assignments was only significant for the White Sea.

Even though there was limited evidence for population structure in ringed seals, genetic distance (F_{ST}) was significantly correlated to geographic distance (r = 0.56566, p = 0.01698). However, when the White Sea comparisons were removed, the correlation was lost (r = 0.12972, p = 0.33367) indicating that most of the correlation is driven by the large genetic and geographic distances between the White Sea and other sampled areas.

Model-based clustering

Structure (version 2; Pritchard *et al.*, 2000) was used to assess population structure in bearded and ringed seals without using information about the geographic origin of samples. All samples were used to assess the probability of the data (X) given one to ten clusters (K). Assuming a uniform prior probability on K, the likelihood of the data [ln (Pr X|K)] at different values of K was used to calculate the probability of each K [(Pr K|X)]. The bearded seal data supported a value of K=2 with a probability of 1.00 (Table 4-8). Under the assumption of two clusters, the inferred ancestry of individuals from the six sampled regions was split between the Bering Sea samples (Gulf of Anadyr and St. Lawrence Island) and the remaining samples in the Beaufort Sea, Labrador Sea, Qaanaaq, and Svalbard (Table 4-9). However, using a less supported model with four clusters, evidence was found for a division between the Beaufort Sea and the eastern samples (Labrador, Qaanaaq, and Svalbard). Additionally, individuals sampled in the Beaufort Sea had greater ancestry in the eastern cluster (0.202), when compared to the Bering Sea (0.149) as evidenced from the results of the assignment test.

For ringed seals, the probability of the data was highest for K=1 (Table 4-8). At higher numbers of clusters (> 1), inferred ancestry was split equally within individuals and within sampled areas. Therefore, no evidence for population structure was found, even for the White Sea sample that showed differentiation using other methods

Discussion

The predictable location of breeding colonies of northern phocid species that breed on land has meant that large numbers of individuals can be observed regularly. The behaviour of marked seals, and individual movement patterns, inferred from satellite telemetry, have led to predictions about the degree of population structure likely to characterize these species. Grey seals aggregate annually to give birth and mate in predictable locations (Anderson *et al.*, 1975; Boness and James, 1979). Although large movements of individuals have been recorded outside the breeding season (Hammond *et al.*, 1993) adult seals show high levels of breeding site fidelity (Pomeroy *et al.*, 1994). The return of large numbers of individuals to specific colonies has led to genetic differentiation between breeding areas that can be detected on a relatively small scale using microsatellite markers (Allen *et al.*, 1995). Likewise, tagging studies in European harbour seals have shown that most individuals have high rates of site fidelity throughout the year (Thompson and Miller, 1990). However, around 20% of pups may move distances of up to 500 km in short periods of time (Thompson *et al.*, 1994). Most of these movements do not constitute effective gene flow and, given the large amount of genetic differentiation found using microsatellite markers (Goodman, 1998), this indicates that dispersing individuals do not recruit to new breeding populations. Therefore, microsatellite markers have shown genetic differentiation in both land breeding species of phocines at small geographic scales. These studies expand on earlier work using mitochondrial DNA that identifies separate stocks of harbour seals and grey seals in the eastern and western Atlantic, and harbour seals in the eastern and western Pacific (Boskovic *et al.*, 1996; Stanley *et al.*, 1996).

Excluding the harp seal (*Pagophilus groenlandicus*) and hooded seal (*Cystophora cristata*), the other northern ice breeding seals do not aggregate in large numbers at any time during the year. For the remaining species, studies of movements, either by satellite telemetry or mark-recapture methods, provide only limited information about individual site fidelity and movements. The use of molecular markers to identify population structure is an obvious alternative method of stock identification, and may be of particular use when large numbers of individuals cannot be studied using traditional

methods. Despite the potential for the use of genetic techniques in identification of population structure, to date these methods have only been used in a limited number of northern ice breeding phocids.

Studies using allozymes, mutlilocus DNA fingerprinting, and mitochondrial cytochrome B sequences have been used to identify separate northeastern and northwestern stocks of harp seals (Meisfjord and Sundt, 1996; Perry *et al.*, 2000), and suggest that while extensive mixing occurs between the two major whelping areas within each region, rates of gene flow across the Atlantic Ocean are low. Population structure in spotted seals (*Phoca largha*) has been investigated using a small number of mitochondrial DNA sequences from seals sampled throughout the species range. The phylogeny of these mtDNA sequences was not consistent with geographic structure (O'Corry-Crowe and Westlake, 1997). On a smaller geographic scale, no evidence for population structure was found among spotted seals sampled in three locations around Hokkaido, Japan (Mizuno *et al.*, 2003). Recently microsatellite markers have been used to examine the evolutionary history of ringed seals in the Baltic Sea (Palo *et al.*, 2001). Seals sampled in the Baltic Sea were only weakly differentiated from individuals sampled in the Arctic, favoring a scenario of high rates of recurrent gene flow between these regions.

Here, we expand on the limited number of population genetic studies in northern ice breeding seals by reporting on genetic structure of bearded and ringed seals. High levels of genetic differentiation were found among most of the six sampled areas of bearded seals. The two populations sampled in the Bering Sea were not differentiated from each other, and there was evidence for limited gene flow among populations sampled in the Atlantic Ocean basin. The lack of differentiation between individuals sampled at St. Lawrence Island and in the Gulf of Anadyr may be explained by the relatively small geographic distance between them, suggesting that individuals may move among population separated by less than 500 km. However, in this region bearded seals migrate annually, moving north with the edge of the pack ice in summer followed by a retreat southward in the winter with the advancing ice sheet. Extensive mixing may occur as seals move and pass through the Bering Straight during these migrations. Strong differentiation was found between the two sampled areas in the Bering Sea and the

remaining sampled areas (Beaufort Sea, Qaanaaq, Labrador Sea, and Svalbard). Genetic distance between these two general areas was greater than expected from the regression of genetic and geographic distance, and suggests that a barrier to gene flow exists somewhere between these regions. Although this could be taken as support for the existence of two subspecies in bearded seals (Manning, 1974), the data here are weak and do not identify the distribution of these subspecies. Evidence for individual movement between the Bering and Beaufort Seas would suggest that gene flow does occur across this barrier, and that the existence of subspecies is doubtful (Kosygin and Potelov, 1971). Significant differentiation was found between areas sampled on either side of the Canadian Arctic island archipelago. Genetic distance was greater between the Beaufort Sea sample and the three sampled locations east of the Canadian archipelago, than it was for comparisons among the later. This suggests that these islands, and the heavy ice that forms in the channels between them, may be a barrier to movements. Among the sampled areas in the Atlantic Ocean, Qaanaaq and the Labrador Sea were not significantly differentiated. However, both were differentiated from the Svalbard sample. Genetic distances among these populations were lower than that observed in other comparisons, suggesting that movements among these populations are more common.

The pattern of population structure presented here supports the contention that bearded seals are relatively sedentary and do not move extensively between breeding areas. Although long distance movements may occur, they are likely restricted to young animals (Gjertz *et al.*, 2000), and may occur post-weaning during times when ice extent is minimal. Upon recruitment to a new area, the preference for pack ice in areas above shallow water (McLaren, 1962) and the reluctance (or inability) of bearded seals to maintain breathing holes in areas of heavy ice, movements are restricted to areas that have open water throughout the year.

In contrast, little evidence for genetic differentiation between sampled areas was found in ringed seals. Except for comparisons to individuals sampled in the White Sea, genetic distances among sampled regions were small and not significantly correlated to geographic distance. This evidence contrasts with the idea that ringed seals are sedentary (McLaren, 1958b) and, although adults that establish breeding territories in regions of prime breeding habitat may have site fidelity (Smith and Hammill, 1981), it indicates

extensive gene flow among regions. The fact that immature and young breeding animals are excluded from good breeding habitat indicates that this type of habitat may be limited. Additionally, it is suspected that ringed seals may also breed in heavy pack ice regions (Finley *et al.*, 1983; Wiig *et al.*, 1999) which may indicate that ringed seals are more generalist in their habitat preference than has been traditionally thought. The vast majority of research on ringed seal pupping and breeding has been done in the fast ice, and the potential for extensive breeding in other areas, including pack ice, has not been quantitatively explored (Reeves, 1997).

If areas of consolidated annual ice with good snow cover (Smith, 1987) in fjords and land-fast ice between islands and coastlines (McLaren, 1958b) are indeed the preferred breeding habitat of ringed seals, then younger animals, which are excluded from these areas, must use less preferred habitat for breeding and may move extensively in search of 'better' habitat. Much evidence has now accumulated to suggest that young ringed seals undertake long distance movements (Smith, 1987; Kapel *et al.*, 1998; Teilmann *et al.*, 1999). Therefore, while a small number of older breeding individuals may show site fidelity, it appears that younger animals can move extensively throughout regions and settle away from their natal area, resulting in high levels of gene flow and limited genetic differentiation between regions. The extensive individual movements of ringed seals that have been observed throughout the Arctic with the resulting lack of population structure may be facilitated by their ability to maintain breathing holes in relatively thick ice, and a broad tolerance for different ice habitats.

In conjunction with previous studies of population structure in northern ice breeding phocids, the results presented here may be used to identify patterns of population structure among the phocine species. The evolutionary origins of the phocines, their phylogenetic relationships, and current species distributions may, in part, explain the levels of contemporary genetic structure. It is likely that the ancestor of the northern seals radiated in the Arctic region during the Pleistocene following isolation in the Parathethys during the Miocene (Ray, 1976). The evolutionary history of the phocines during this time is undoubted complex, but was likely was influenced by subsequent northward and southward expansions in range during periods of global cooling and warming. During warmer periods, greater ease of exchange across the Arctic

may have led to the current geographic distribution of species, with some species (ribbon and largha seals) confined to the Pacific Ocean basin, and others (harp and hooded seals) distributed only in the Atlantic. Given that the deepest branching modern phocine, the bearded seal, is adapted to cold climate and ice breeding (Perry *et al.*, 1995), it is reasonable to suggest that the ancestor of the phocines shared these characteristics and may also have been relatively sedentary, in part, due to an inability to move through areas of heavy ice. Current patterns of genetic differentiation in harp seals would suggest that these traits have been retained in later evolving species. The observed divergence of northeastern and northwestern Atlantic stocks of harp seals (Meisfjord and Sundt, 1996; Perry *et al.*, 2000) would suggest that extensive movements do not occur through the heavy ice of the north Atlantic, but that much exchange occurs between whelping areas, in areas of ice with more open water on either side of the Atlantic Ocean. The lack of contemporary genetic structure in spotted seals (O'Corry-Crowe and Westlake, 1997; Muzino *et al.*, 2003) may be due to their somewhat limited geographic distribution or the fact that heavy ice does not form throughout most of their range, allowing for extensive movements between breeding areas.

If the phocine ancestor was adapted to ice breeding, the presence of land breeding behaviour in harbour and grey seals must be a secondarily evolved trait (Perry *et al.*, 1995). Adaptation to land breeding has resulted in high level of genetic differentiation between geographically close breeding areas in these species (Allen *et al.*, 1995; Goodman, 1998), because individuals show high levels of fidelity of specific regions for breeding. Ringed seals, in contrast have become so adapted to the ice environment that they can move extensively through both pack ice and more dense ice types, resulting in panmixia throughout most of their Arctic range.

Predictions about population structure in ice breeding seals that have not yet been studied, can be made using the framework presented above. Hooded seals (*Cystophora cristata*) are distributed in a similar fashion to harp seals. They prefer areas of drifting pack ice above deep water and generally avoid areas of heavy ice (King, 1983). Additionally, they breed in loose aggregations in the same areas as harp seals. Given these characteristics it is likely that stocks of hooded seals in the eastern and western Atlantic Ocean will be genetically differentiated, but that high levels of interchange will

be observed within these regions. Ribbon seals (*Histriophoca fasciata*) have a similar distribution to spotted seals. Therefore, given their preference for breeding in floating ice, their inability to maintain breathing holes in thick ice, and their limited distribution it is likely that only limited population structure has developed in this species.

The examination of population structure in bearded and ringed seals presented here indicates that certain life history traits have been important in the development of genetic differentiation in Arctic phocids. These traits and information about the evolutionary history of the Phocinae can be used to partially explain the current levels of genetic differentiation found in ice and land breeding species. In addition, the results of this study provide estimates of genetic variation and differentiation in bearded and ringed seals that may be used to evaluate future change in the Arctic ecosystem.

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Table 4-1. Geographic location, two letter identifier, sample size, date of sampling, and type of sample for a) bearded seals and b) ringed seals.

a) bearded

Population	ID	N	Sample Type	Date
Gulf of Anadyr, Russia	GA	25	Muscle	1991
St. Lawrwnce Island, USA	SL	28	Muscle	1991
Beaufort Sea, Canada	BF	16	Muscle/Bone	1973-1996
Labrador Sea, Canada	LD	16	Muscle	1995-1999
Qaanaaq, Greenland	QA	16	Skin	1990s
Svalbard, Norway	SV	18	Muscle/Blood	1990s

b) ringed

Population	ID	N	Sample Type	Date
Alaska, USA	AK	23	Muscle	1984
Holman, NWT, Canada	H4	21	Muscle	1994
Holman, NWT, Canada	H6	20	Muscle	1996
Holman, NWT, Canada	H8	30	Muscle	1998
Holman, NWT, Canada	H9	11	Skin	1999
Minto Inlet, NWT, Canada	MN	25	Skin	1999
Arviat, Nunavut, Canada	AV	28	Muscle	1998
Iqaluit, Nunavut, Canada	IQ	29	Muscle	1998
Grise, Fjord, Nunavut, Canada	GF	31	Muscle	1998
Qaanaaq, Greenland	QA	30	Muscle	1998
Svalbard, Norway	SV	30	Muscle	1990s
White Sea, Russia	WS	25	Muscle	1998

Table 4-2. Microsatellite loci from Davis *et al.* (2002), Allen *et al.* (1995), and Goodman (1997, 1998) used to genotype bearded and ringed seals.

Locus	Lw-7	Hl-8	Hl-15	Hl-16	Hl-20	Lc-6	Lc-18	Lc-28	Hg4.2	Hg6.1	Hg6.3	Hg8.9	Hg8.10	SGPV10	SGPV11	Total
Bearded Seal	√	√		√	√		√	√	√	√	√	√	√	√	√	13
Ringed Seal	√	√	√	√		√	√		√	√	√		√		√	11

Table 4-3. Results of tests of disequilibrium including Hardy-Weinberg equilibrium, linkage disequilibrium, and heterogeneity of allele distributions for; a) bearded seal, and; b) ringed seal. Observed number of tests returning significant results and the number expected due to Type I error if null hypothesizes are correct. Two values of significance are shown. Bonferroni corrections were made for multiple tests with an initial value of $\alpha = 0.05$ (adjusted values given in square brackets).

a)

	Individual HW		Global HW (pops)		Global HW (loci)		Linkage disequilibrium		Allele distributions	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
P < 0.05	3	3.90	2	0.30	1	0.65	20	23.40	13	0.75
P < 0.01	1	0.78	0	0.06	0	0.13	13	4.68	13	0.15
Bonferroni	0	[0.00064]	0	[0.00833]	0	[0.00390]	0	[0.00011]	13	[0.00333]

b)

	Individual HW		Global HW (pops)		Global HW (loci)		Linkage disequilibrium		Allele distributions	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
P < 0.05	14	6.60	2	0.60	2	0.55	17	33.00	17	3.30
P < 0.01	5	1.32	1	0.12	2	0.11	4	6.60	11	0.66
Bonferroni	0	[0.00038]	0	[0.00420]	0	[0.00450]	1	[0.00008]	11	[0.00076]

Table 4-4. Genetic variation of loci used to genotype a) bearded seals and b) ringed seals. Diversity expressed as allelic diversity (A) and expected heterozygosity (H_E). The range of estimates across populations is given in parenthesis. Species source of microsatellite markers is also shown.

a) bearded

Locus	Source Species	A	H_E
Lw-7	Weddell seal	4.00 (3-5)	0.49 (0.29-0.65)
Hl-8	leopard seal	7.50 (4-13)	0.78 (0.65-0.86)
Hl-16	leopard seal	9.33 (3-18)	0.79 (0.68-0.93)
Hl-20	leopard seal	6.00 (5-7)	0.73 (0.71-0.77)
Lc-18	crabeater seal	3.17 (2-5)	0.57 (0.47-0.61)
Lc-28	crabeater seal	5.83 (4-8)	0.69 (0.57-0.86)
Hg4.2	grey seal	7.17 (3-10)	0.68 (0.50-0.86)
Hg6.1	grey seal	8.00 (6-9)	0.76 (0.71-0.81)
Hg6.3	grey seal	5.33 (4-7)	0.68 (0.58-0.75)
Hg8.9	grey seal	5.83 (4-9)	0.77 (0.73-0.84)
Hg8.10	grey seal	2.33 (2-3)	0.52 (0.47-0.57)
SGPV10	harbor seal	2.33 (2-4)	0.22 (0.06-0.50)
SGPV11	harbor seal	4.17 (3-5)	0.60 (0.47-0.74)

b) ringed

Locus	Source Species	A	H_E
Lw-7	Weddell seal	4.67 (3-8)	0.37 (0.19-0.61)
Hl-8	leopard seal	13.50 (11-16)	0.91 (0.86-0.94)
Hl-15	leopard seal	19.08 (10-24)	0.94 (0.91-0.96)
Hl-16	leopard seal	13.92 (9-18)	0.91 (0.86-0.93)
Lc-6	crabeater seal	12.67 (10-16)	0.87 (0.84-0.91)
Lc-18	crabeater seal	11.42 (6-15)	0.85 (0.76-0.90)
Hg4.2	grey seal	16.17 (12-18)	0.92 (0.90-0.94)
Hg6.1	grey seal	17.08 (13-21)	0.91 (0.82-0.94)
Hg6.3	grey seal	11.33 (10-12)	0.88 (0.85-0.92)
Hg8.10	grey seal	9.25 (6-13)	0.73 (0.67-0.79)
SGPV11	harbor seal	13.33 (11-17)	0.87 (0.79-0.93)

Table 4-5. Genetic diversity across populations of; a) bearded seals, and; b) ringed seals. Diversity is expressed as allelic diversity (A), expected heterozygosity (H_E), and observed heterozygosity (H_O).

a) bearded

Population	ID	N	A	H_E	H_O
Gulf of Anadyr	GA	25	7.08	0.66	0.64
St. Lawrence Island	SL	28	7.54	0.69	0.62
Beaufort Sea	BF	16	4.62	0.65	0.61
Labrador Sea	LD	16	4.69	0.61	0.60
Qaanaaq	QA	16	4.85	0.62	0.58
Svalbard	SV	18	4.00	0.60	0.66
Total (Mean for A, H_E, H_O)	6	119	5.46	0.64	0.62

b) ringed

Population	ID	N	A	H_E	H_O
Alaska	AK	23	13.45	0.83	0.83
Holman, NWT (1994)	H4	21	12.09	0.81	0.77
Holman, NWT (1996)	H6	20	12.09	0.86	0.88
Holman, NWT (1998)	H8	30	14.09	0.85	0.80
Holman, NWT (1999)	H9	11	9.55	0.85	0.81
Minto Inlet, NWT	MN	25	12.91	0.81	0.81
Arviat, Nunavut	AV	28	14.09	0.84	0.83
Iqaluit, Nunavut	IQ	29	14.45	0.84	0.80
Grise Fjord, Nunavut	GF	31	13.82	0.83	0.82
Qaanaaq, Greenland	QA	30	14.18	0.85	0.85
Svalbard, Norway	SV	30	13.18	0.82	0.81
White Sea, Russia	WS	25	11.45	0.78	0.79
Total (Mean for A, H_E, H_O)	12	303	12.95	0.83	0.82

Table 4-6. Genetic distance between study populations of; a) bearded seals, and; b) ringed seals. F_{ST} (× 100) lower matrix, D_{LR} upper matrix. F_{ST} estimates that are significantly greater than zero are shown in bold. Rectangles highlight distances within three population clusters in bearded seals.

a) bearded

	GA	SL	BF	LD	QA	SV
GA		0.12	4.49	5.40	4.63	8.31
SL	-0.20		4.59	6.54	5.38	9.16
BF	**8.39**	**7.47**		2.52	2.24	2.87
LD	**7.70**	**7.63**	**6.28**		0.30	1.14
QA	**6.90**	**6.43**	**3.00**	1.33		1.62
SV	**12.33**	**11.95**	**6.17**	**2.08**	**3.23**	

b) ringed

	AK	H4	H6	H8	H9	MN	AV	IQ	GF	QA	SV	WS
AK		0.69	-0.25	-0.32	-0.55	0.24	-0.58	0.27	0.07	-0.01	0.10	3.14
H4	0.41		0.42	0.16	-0.57	0.24	0.63	0.18	-0.08	-0.18	0.68	2.82
H6	0.14	0.61		-0.20	-0.10	0.50	0.08	-0.22	0.06	0.31	0.41	3.92
H8	0.53	0.31	-0.16		-0.90	0.15	-0.81	0.44	0.22	-0.33	0.56	3.95
H9	0.00	-0.73	-0.24	-0.64		-0.11	-0.90	-0.12	-0.42	-1.42	0.30	3.11
MN	0.18	0.48	0.20	0.30	0.17		0.52	0.03	0.85	0.56	0.83	4.21
AV	0.14	0.37	0.04	-0.39	-0.55	0.43		-0.51	-0.06	-0.57	-0.18	2.73
IQ	0.42	-0.13	-0.05	-0.07	-0.02	0.25	-0.01		0.91	-0.04	-0.04	4.19
GF	0.24	-0.20	0.00	0.43	-0.50	0.52	0.17	0.24		-0.28	1.01	3.02
QA	0.51	0.30	0.01	0.17	-0.64	0.64	0.11	0.29	-0.01		0.44	2.77
SV	0.42	-0.14	0.26	0.23	-0.36	0.21	0.06	-0.01	0.38	0.28		4.26
WS	**2.93**	**2.00**	**2.69**	**3.18**	**2.07**	**2.33**	**3.08**	**2.43**	**1.76**	**2.46**	**2.59**	

Table 4-7. Results of the assignment test for; a) bearded seals, and; b) ringed seals. Rows contain individuals sampled in each population, while columns indicate the populations to which individuals were assigned. Values in bold indicate groups where significant numbers of individuals assigned to their nominal population.

a) bearded

	GA	SL	BF	LD	QA	SV	Total
GA	**14**	11					25
SL	11	**16**	1				28
BF		1	**11**	1	1	2	16
LD			2	**8**	4	2	16
QA			1	2	**10**	3	16
SV			1	4	2	**11**	18

b) ringed

	AK	H4	H6	H8	H9	MN	AV	IQ	GF	QA	SV	WS	Total
AK	0	1	3	1	1	5	4	1	3	1	2	1	23
H4	0	1	1	6	0	4	1	1	4	2	1	0	21
H6	4	1	3	2	0	2	2	1	3	0	2	0	20
H8	2	3	1	2	5	3	5	1	3	2	3	0	30
H9	0	1	1	2	0	1	1	1	0	3	1	0	11
MN	2	3	2	2	0	3	2	3	5	3	0	0	25
AV	3	0	4	2	0	0	4	4	4	4	2	1	28
IQ	0	4	2	3	1	3	4	4	1	2	5	0	29
GF	0	4	3	3	0	0	5	0	5	7	3	1	31
QA	0	3	2	1	4	3	2	4	5	4	0	2	30
SV	2	2	3	1	3	1	2	5	1	3	7	0	30
WS	1	1	1	0	1	0	3	0	1	1	2	**14**	25

Table 4-8. Results of cluster analysis using Structure. The log likelihood [ln Pr (X|K)] of the data given a specified number of clusters (K), and posterior support for given Ks [Pr (K|X)] assuming a uniform prior on K. High probabilities for given Ks are shown in bold

	Bearded Seal		Ringed Seal	
K	ln Pr (X|K)	Pr (K|X)	ln Pr (X|K)	Pr (K|X)
1	-4613.2	< 0.0001	**-15914.2**	**1.0000**
2	**-4342.8**	**1.0000**	-16038.8	< 0.0001
3	-4423.9	< 0.0001	-16213.5	< 0.0001
4	-4438.1	< 0.0001	-19917.0	< 0.0001
5	-4538.4	< 0.0001	-19794.0	< 0.0001
6	-4634.9	< 0.0001	-20090.7	< 0.0001
7	-4609.0	< 0.0001	-29433.5	< 0.0001
8	-4774.3	< 0.0001	-15924.4	< 0.0001
9	-4776.0	< 0.0001	-27914.2	< 0.0001
10	-5423.9	< 0.0001	-16468.3	< 0.0001

Table 4-9. Proportion of ancestry inferred in each of either two or four clusters determined from Structure analysis in bearded seals. For each sampled area, the highest inferred ancestry in any cluster is highlighted in grey.

	K = 2 Clusters		K = 4 Clusters		
Population	1	2	1 + 2	3	4
GA	0.943	0.057	0.920	0.063	0.018
SL	0.950	0.050	0.926	0.057	0.017
BF	0.208	0.792	0.149	0.649	0.202
LD	0.043	0.957	0.071	0.210	0.718
QA	0.071	0.929	0.088	0.356	0.556
SV	0.021	0.979	0.028	0.115	0.857

Figure 4-1a. Sampling location of bearded seals (N = 6). Two-letter codes refer to the geographic names of the location of sampling (see Table 4-1a).

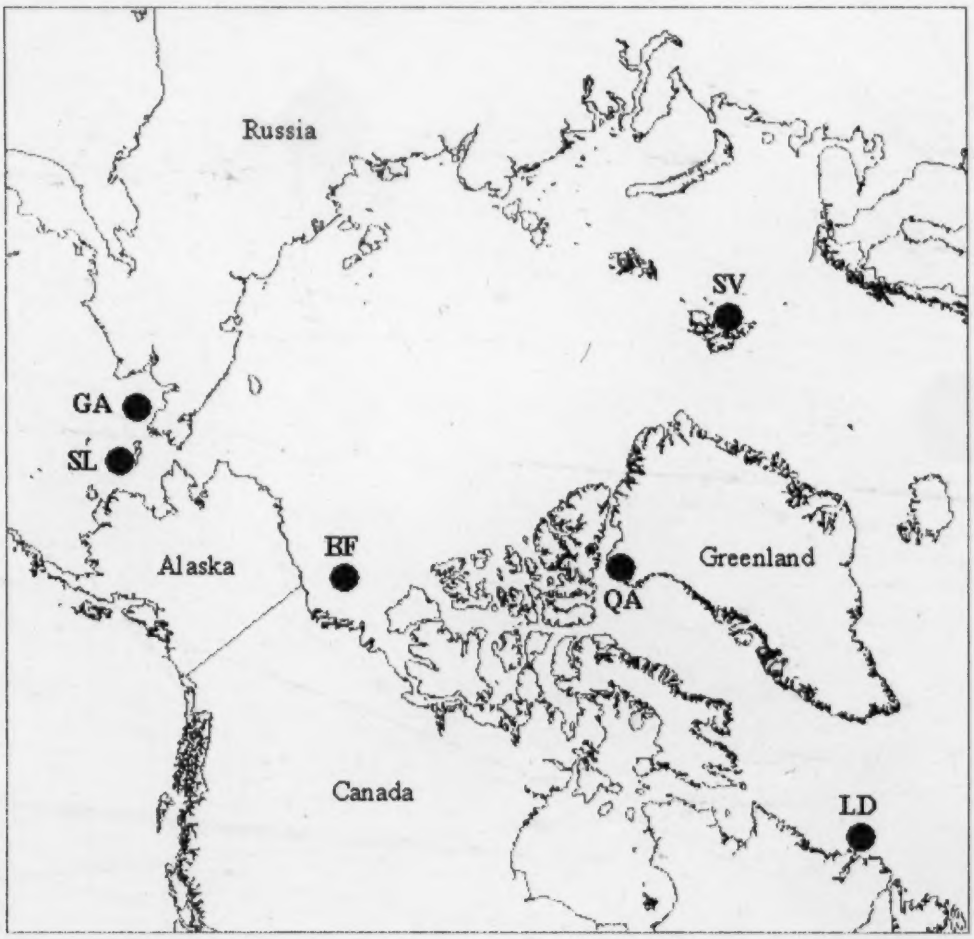


Figure 4-1b. Sampling location of ringed seals (N = 12). Two-letter codes refer to the geographic names of the location of sampling (see Table 4-1b).

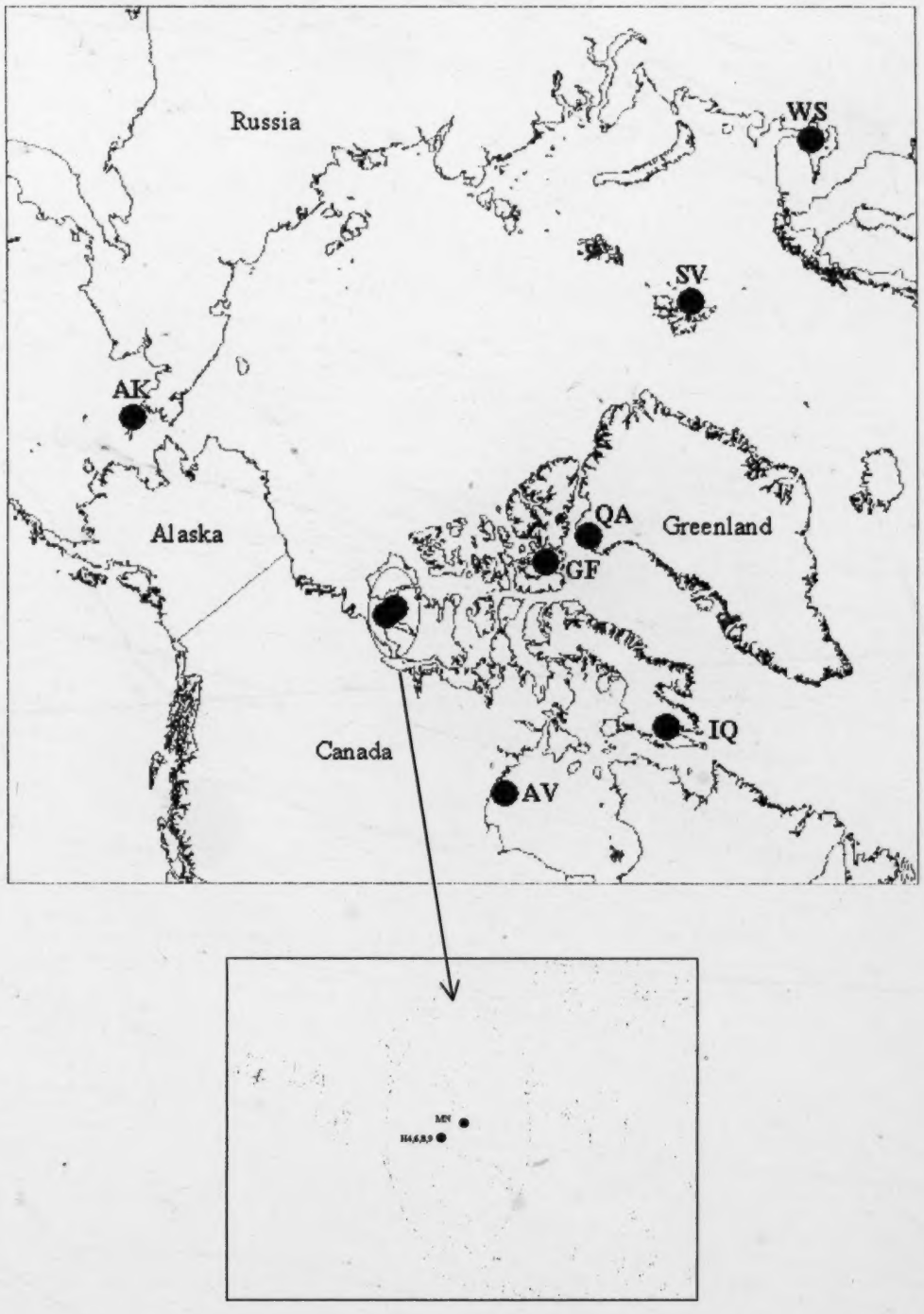
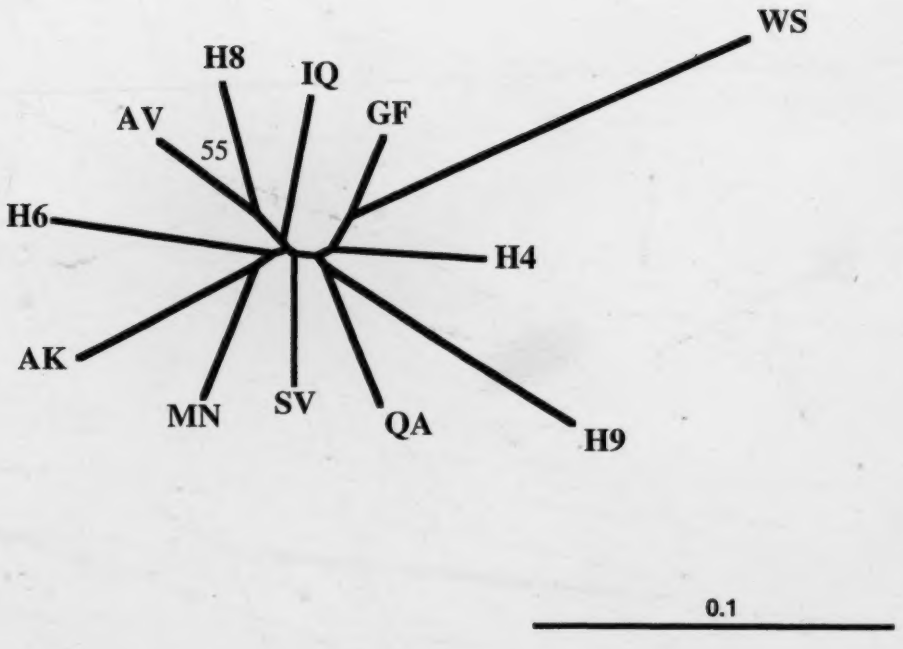


Figure 4-2. Fitch-Margoliash trees of Nei's standard distance (D_S) between study populations for a) bearded seals and b) ringed seals. Bootstrap values are shown for clades with greater than 50% support.

a)

b)



Chapter 5
General Conclusions

Introduction

Thirty-three species of true seals (Phocidae), sea lions and fur seals (Otariidae), and walrus (Odobenidae) are distributed worldwide. Those species that are best known to man occur in tropical, temperate, and boreal regions. Most species that occur in these areas are highly gregarious and can be observed in large groups at predictable locations where they haul-out for parturition and breeding. Some also display marked sexual dimorphism and have polygynous mating systems, where dominant males establish large harems and are successful in mating with most females. Due to the fact that the pinnipeds that are most familiar to us share many of these characteristics, there is sometimes a misconception that all pinnipeds must possess these traits. Another implicit, although often unstated assumption, about pinniped behaviour is that because seals return each year to the same areas to breed, populations must be geographically structured. In fact, nearly half of all pinniped species are not gregarious, display little or no sexual dimorphism, and do not have polygynous mating systems. Some of these species inhabit polar regions and use ice as a substrate for parturition after which they mate in the water. It is not known how structured populations are in these species. Much research has been focused on the assessment of mating and population structure in tropical, temperate, and boreal pinniped species because their habitat is easily accessible and large numbers of individuals can be observed in predictable locations. However considerably less information is available about pinniped species that inhabit polar regions (reviewed by Hoelzel, 1997).

The purpose of the work presented in this thesis was to examine genetic diversity in seal species from the Antarctic and Arctic in the hope of gaining a greater understanding of the factors that influence population differentiation in ice breeding seals (Chapters 3 & 4). A simple hypothesis was developed, as an extension of theories relating to the development of mating structure in pinnipeds, to predict the development of population structure based on breeding habitat. It was recognized that closely related

species may share characteristics that either promote or deter the development of population structure, even though they occupy different habitats. In order to account for such an occurrence, the phylogeny of the extant Phocidae was investigated using complete mitochondrial DNA coding sequences (Chapter 2). Given the pattern of genetic differentiation observed for each species, phylogenetic relationships, known life history characteristics, and breeding habitat it may be possible to explain the observed patterns of population structure among northern and southern ice breeding seals.

Breeding habitat and population structure

Differences in breeding habitat preference may be related to the development of different mating systems in pinnipeds (Bartholomew, 1970; Stirling, 1983). It is also likely that the development of population structure is directly related to levels of breeding site fidelity. This is because the degree of site fidelity may vary due to the type of habitat used for parturition. For many species individuals are solitary outside of the breeding season and move extensively during pelagic foraging. However, during the breeding season individuals interact socially during the brief, yet highly temporally synchronized, mating season. Population structure will develop when breeding occurs among related individuals that return to breeding sites with fidelity. In contrast, if individuals show only general preferences for particular types of habitat, and not for specific sites, extensive mixing of individuals will result in limited population differentiation.

Three general types of habitat are used for parturition and mating by phocid seals (Stirling, 1983). These are terrestrial (land), pack ice (drifting ice) and land-fast ice (ice contiguous with land or islands). For species that use terrestrial breeding areas, the predictability and stability of this habitat may cause individuals to show high levels of site fidelity. In contrast, the unlimited and uniform characteristics of the pack ice breeding habitat may lead to large inter-annual movements of individuals throughout their widely spaced breeding ranges. Cracks that form in predicable locations influence where breathing holes occur naturally, or may be more easily maintained, in the land-fast ice habitat of polar regions. Even so, annual variations in ice conditions make this habitat less predictable than terrestrial pupping sites. Therefore species that use land-fast ice for

parturition and mating may show levels of site fidelity and population structure intermediate to pack ice and terrestrial breeding species.

Population structure in southern phocids

Genetic differentiation between groups of individuals sampled in different geographic locations has been investigated in six of eight 'southern' phocids (Monachinae). The results of these studies generally support ideas about the effect of breeding habitat and site fidelity on the development of population structure. Southern elephant seals, which breed terrestrially on subantarctic islands, show high levels of genetic differentiation between breeding colonies (Slade *et al.*, 1998; Hoelzel *et al.*, 2001). Studies of genetic variation in the northern elephant seal have revealed extremely low variation at all loci tested (Hoelzel, 1997). Therefore it has been impossible to investigate current population structure in this species. The aquatically mating Hawaiian monk seal, which breeds in the water near beaches on the northwestern Hawaiian Islands, is also genetically depauperate, but a recent study using microsatellite markers suggests differentiation between distant breeding colonies (Kretzmann *et al.*, 2001). Similar studies have not been performed on the Mediterranean monk seal. Weddell seal breeding colonies found in the land-fast ice of the Antarctic continent are genetically differentiated, however Ross, leopard and crabeater seals that use the pack ice surrounding Antarctica as a breeding substrate display no genetic differentiation between widely spaced localities (Davis *et al.*, 2000; Chapter 3). Of the monachine species examined to date, terrestrial land breeding species show high levels of genetic differentiation between breeding colonies, aquatic mating land dwelling species and fast-ice breeding species show moderate levels of genetic structure and pack ice breeding species show no differentiation between geographic areas.

Evolution of population structure in the southern phocids

Although there is much debate over the geographic location of the origin of the monachine lineage (Ray, 1976; de Muizon, 1982) and even greater confusion regarding

the radiation of the group (see Berta, 2002), it is generally agreed that the monachine ancestor was found in warm waters at low latitude (de Muizon, 1982). It was probably much like the monk seals of today and mated in the water near ice-free beaches. Population structure has been found in Hawaiian monk seals (Kretzmann *et al.*, 2001) which breed in the water near land however no studies of genetic differentiation have been performed on the Mediterranean monk seal. The elephant seals (*Mirounga spp.*) evolved from the monachine stock and are currently characterized by land breeding in terrestrial areas. Large amounts of genetic differentiation exist between southern elephant seal breeding colonies (Slade, 1998; Hoelzel, 2001). The ancestor of the Antarctic seals moved to high latitudes during a single invasion and radiated in the southern ocean. The first seals to venture into the Antarctic region were probably exposed to environmental conditions similar to those that exist today and this provided the mechanism for rapid change in the Lobodontines. One significant change would have been the need to use ice as a platform for parturition. With no barriers to movement around the Antarctic continent, and the vast pack ice field surrounding it, individuals lost fidelity to specific regions for breeding. As a consequence, population differentiation was lost, as is observed in the modern Ross, leopard and crabeater seals (Davis *et al.*, 2000; Chapter 3). The more recent branching Weddell seal adapted to exploit the land-fast ice regions of the Antarctic continent, and now breeds in predictable stable areas. Fidelity of individuals to these areas for breeding has resulted in significant population structure between breeding areas.

Population structure in northern phocids

Population structure has now been investigated in 6 of 10 northern phocid species. Grey seals which breed in terrestrial colonies show significant genetic differentiation even at small geographic scales (Allen *et al.*, 1995). Likewise, colonies of terrestrial breeding European harbour seals are differentiated over short distances (Goodman, 1998). Harp seals, which are a highly gregarious pack ice breeding species, have genetically discernable stocks in the northeastern and northwestern Atlantic (Meisfjord and Sundt, 1996; Perry *et al.*, 2000). Pack ice breeding spotted seals do not display genetic

differentiation across much of their range (O'Corry-Crowe and Westlake, 1997; Mizuno, 2003). Bearded seals, that pup and breed in pack ice, are highly differentiated between geographic regions (Chapter 4). In contrast, ringed seals that are thought to pup and breed in areas of stable predictable land-fast ice display little population structure. Clearly these results suggest that the stability and predictability of breeding habitat and cannot explain the observed patterns of population structure in northern seals.

Evolution of population structure in northern phocids

The evolutionary origins of the phocines, their phylogenetic relationships, and current species distributions may, in part, explain the levels of contemporary genetic structure. It is likely that the ancestor of the northern seals radiated in the Arctic region during the Pleistocene, following isolation in the Parathethys during the Miocene (Ray, 1976). The evolutionary history of the phocines during this time is undoubtedly complex, but was likely was influenced by subsequent northward and southward expansions in range during periods of global cooling and warming. During warmer periods greater ease of exchange across the Arctic may have led to the current geographic distribution of species, with some species (ribbon and largha seals) confined to the Pacific Ocean basin, and others (harp and hooded seals) distributed only in the Atlantic. Given that the earliest branching modern phocine, the bearded seal, is adapted to cold climate and ice breeding (Perry *et al.*, 1995), and shows marked population structure (Chapter 4) it is possible that the ancestor of the phocines shared these characteristics and may also have been relatively sedentary, in part, due to an inability to move through areas of heavy ice. Current patterns of genetic differentiation in harp seals would suggest that these traits have been retained in later evolving species. The observed divergence of northeastern and northwestern Atlantic stocks of harp seals (Meisfjord and Sundt, 1996; Perry *et al.*, 2000) suggests that extensive movements do not occur through the heavy ice of the north Atlantic, but that much exchange occurs between whelping areas, in areas of ice with more open water, on either side of the Atlantic Ocean. The lack of contemporary genetic structure in spotted seals (O'Corry-Crowe and Westlake, 1997; Muzino *et al.*, 2003) may be due to their somewhat limited geographic distribution or the fact that heavy ice does

not form throughout most of their range, allowing for extensive movements between breeding areas.

If the phocine ancestor was adapted to ice breeding, the presence of land breeding behaviour in harbour and grey seals must be a secondarily evolved trait (Perry *et al.*, 1995). Adaptation to land breeding has resulted in high level of genetic differentiation between geographically close breeding areas in these species (Allen *et al*, 1995; Goodman, 1998), because individuals show high levels of fidelity of specific regions for breeding. Ringed seals, in contrast have become so well adapted to the ice environment that they can move extensively through both pack ice and more dense ice types resulting in panmixia throughout most of their Arctic range (Chapter 4).

Summary

The events that have led to the development of population structure in southern and northern seals are clearly complex and have been influenced by historical processes. All species that currently breed on land, or in the water adjacent to it, are characterized by population structure. In contrast, pack ice breeding seals in the Antarctic have limited population structure while many northern pack ice species display regional genetic differentiation. Of the two phocid species that breed in the land-fast ice, Weddell seals in the Antarctic have discernable breeding populations while ringed seals of the Arctic do not. In the Arctic, the processes leading to the evolution of population structure has likely been influenced by a cold adapted, ice breeding ancestor that was unable to move through heavy areas of ice. In contrast, the evolution of pagophilic behaviour in the Antarctic lobodontines has led to the loss of population in most species.

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Chapter 6

Addendum - Dinucleotide microsatellite markers from the Antarctic seals and their use in other Pinnipeds[1]

The inaccessibility and instability of the Antarctic pack ice has limited study of population dynamics in Antarctic seals due to the inability to regularly observe large number of individuals. Likewise, aquatic breeding that cannot be directly observed hampers studies of mating structure. Therefore molecular techniques, such as microsatellite genotyping, may be of particular value in studying population dynamics and mating systems in Antarctic seal species. Microsatellite loci have been developed for the grey seal (Allen *et al.* 1995) and the harbor seal (Goodman 1998, Coltman *et al.* 1996), and have been shown to be polymorphic in northern phocids, but are of limited use in the monachines (Gemmell *et al.* 1997). Therefore in order to obtain loci useful in the Antarctic species, microsatellites were isolated from small insert genomic libraries, of the leopard, crabeater, and Weddell seals. These microsatellite loci are being used to estimate population structure in the four pack ice seal species (e.g. Davis *et al.* 2000) and mating structure and male reproductive success in Weddell seals (e.g. Gelatt *et al.* 2000).

Libraries were constructed using methods similar to those described for humans (Weber and May 1989) and bears (Paetkau and Strobeck 1994). For each library approximately 50 μg of genomic DNA was isolated from muscle tissue by standard methods (Sambrook *et al.* 1989) and digested to completion with Sau3A1. The resulting fragments were electrophoresed on a 1% agarose gel. Fragments of 200-500 bp were excised and isolated from the gel using QIAquick™ spin columns (QIAGEN). Size selected fragments were ligated into BamHI digested dephosphorylated M13mp18RF (Gibco BRL) and transfected into *E.coli* DH5αF' by electroporation (E. coli Pulser™, Bio-Rad). Approximately 5000 recombinant clones from each library were screened by plaque lifts and hybridization to a biotinylated $(GT)_{11}$ oligonucleotide probe, allowing for detection with a nonradioactive detection kit (Gibco BRL). Eighty positive clones from the leopard seal library, 43 from the crabeater seal, and 22 from the Weddell Seal were isolated. Following secondary screening, genomic inserts from 23 leopard seal clones, 23

crabeater seal clones, and 17 Weddell Seal clones were PCR amplified, gel purified, sequenced using ABI dRhodamine chemistry and resolved on a 377 ABI DNA sequencer. Primers were designed, using OLIGO (National Biosciences Inc., Version 4.0) with one primer of each set fluorescently labeled with one of 6-FAM, TET or HEX (ABI) for products containing microsatellite repeats in 20 leopard seal, 16 crabeater seal, and 17 Weddell Seal sequences. Microsatellite PCR for all loci was performed as follows. Total genomic DNA was isolated from skin, muscle or blood using QIAamp™ spin columns (QIAGEN). Approximately 100 ng of DNA was used as template in 15 μL PCR reactions also containing 0.16 μM (2.4 ρmole) of each primer, 120 μM dNTPs, 2 mM $MgCl_2$, 0.3 units of Taq DNA polymerase (isolated as in Engelke *et al.* 1990) and 1X PCR buffer (10 mM Tris buffer, pH 8.8, 0.1% Triton X100, 50 mM KCl and 0.16 mg/ml BSA). Cycling was performed on a Perkin Elmer™ 9600 thermal cycler under the conditions: 1 min at 94 °C, three cycles of 30 sec at 94 °C, 20 sec at 49 °C, and 5 sec at 72 °C, 33 cycles of 15 sec at 94 °C, 20 sec at 50 °C, and 1 sec at 72 °C, final extension at 72 °C for 30 min. PCR products were resolved on a model 373A or 377 DNA Sequencer and analyzed using Genescan™ and Genotyper™ software (ABI).

A total of twenty-four primer pairs amplified strong interpretable banding patterns among the three target species. Seven loci isolated from the leopard seal library were tested for variability on 21 leopard seals captured at Bird Island, South Georgia in 1996. Six crabeater seal loci were tested on 25 crabeater seals sampled in 1994 from the R.V. Nathaniel B. Palmer, and 11 Weddell Seal loci were tested on 96 adult Weddell Seals sampled in 1996 at Big Razorback Island, McMurdo Sound. These 24 loci showed high levels of variability with 3-19 alleles per locus and expected heterozygosity ranging between 33.8 and 92.8% (Table 6-1). One locus, Lw-18, was X-linked as all males (N=28) appeared homozygous, while two loci (Lw-5 and Lw-15) displayed null alleles as determined from mismatches between known mother offspring pairs.

We also tested all primer sets for their ability to amplify homologous microsatellites in 10 other pinniped species and between the focal (crabeater, leopard and Weddell) species (Table 6-2). All 24 loci amplified variable regions in all three of the species used to clone microsatellites. Likewise, 22 of 24 loci amplified variable microsatellite loci in the closely related Ross Seal (*Ommatophoca rossii*). Twenty loci

could be amplified in the Northern elephant seal, although only 6 were variable, indicative of the low variation found in northern elephant seals using other markers. Cross species amplification in 6 species of northern phocids were largely successful with an average of 19 loci amplifying in these species. The more taxonomically distant fur seal (*Arctocephalus fosteri*) and walrus (*Odobenus rosmarus*) amplified only 12 and 10 loci respectively.

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Table 6-1. Size range, repeat motif of sequenced allele, primer sequences (5' to 3'), Genbank accession numbers, and diversity statistics of 24 Antarctic pack ice seal microsatellite loci. Number of alleles (N_A), and observed heterozygosity (Ho)were estimated from 96 adult Weddell seals (for Lw-X loci), 21 leopard seals (for Hl-X loci) and 25 crabeater seals (for Lc-X loci). H_E was calculated using the formula $(1-\Sigma P_i^2)/(n-1)$. Observed size range of product is given in base pairs. Labeled (L) primers are indicated by the notationX_F, X_T, or X_H denoting 6-FAM, TET or HEX fluorescent dye groups (ABI), respectively. Unlabelled primers (U) have been "tailed" with GTTT to promote non template mediated adenylation of the PCR product (Brownstein *et al.* 1996), for loci Lw-20, Hl-14, and Lc-13. * indicates X-linked.

Locus	Size Range	Repeat Motif	N_A	H_O	H_E	Primer Sequences	Accession Number
Lw-4	125-195	$(GT)_{17}$	14	0.9063	0.8540	L: X_TTCCCAGAAGACCTACTCC U: ATTCCTTTCCTGCGTATC	AF140590
Lw-5	96-124	$(GT)12T(G)_6$	13	0.8229	0.8810	L: X_TGGAGTCCCTGAAATCACC U: GGGACCAAACAAAACCTG	AF417688
Lw-7	159-173	$(GT)_{16}$	7	0.6523	0.6684	L: X_HTGGGCTTTCTACAGTTC U: ACATAACTCAAGGGACAA	AF140591
Lw-8	103-107	$(GT)_{11}$	3	0.4167	0.4025	L: X_HCCTCTTTTCCTCTCTCTT U: CAATGTGGATGGAGTAAA	AF417689
Lw-9	96-104	$(GT)_3ACA(GT)_{10}$	5	0.3125	0.4251	L: X_FTACATTCCACAAAGTTG U: GTATATTAAAAATCCTAAAG	AF417690
Lw-10	110-138	$(GT)_{25}$	14	0.9063	0.8792	L: X_TAACACTAGCCCTGACTTC U: TTACAGAGCAGGAGTTCA	AF140592
Lw-11	157-187	$(GT)_{26}$	15	0.8854	0.8683	L: X_FCTCTCCCTCTCACCTTCC U: GGCAAATGAGGTGATGTC	AF140593
Lw-15	138-152	$(GT)_{11}$	8	0.7604	0.8052	L: X_FGATCTCTCTCTCTTTCAC U: CTGTAACTTCTCCAAACA	AF417691
Lw-16	161-175	$(GT)_{11}$	7	0.7604	0.7767	L: X_TCACTCCCCCACTGCTTGT U: ATTAGTTGCAATTTTGAGACACTC	AF140594
Lw-18*	82-120	$(GT)_{17}(AG)_{11}$	9	0.6765	0.6990	L: X_FCACACCCGCCAACTCAT U: TTTACCTCCAATTCTTCAGAT	AF140596
Lw-20	122-146	$(GT)_{20}$	12	0.8229	0.8091	L: X_HGACTCTTGCCCCCTTCAG U: GTTTCACAGACCTGCCTCTAGTG	AF140595

Table 6-1. *Continued*

Locus	Size Range	Repeat Motif	N_A	H_O	H_E	Primer Sequences	Accession Number
Hl-2	110-114	$(GT)_9C(GT)_5$	3	0.3810	0.3821	L: X_FCAAACACCACTATTTCCCT U: AGGTTGTGGTCTGAAGAAT	AF417692
Hl-4	125-133	$(GT)_{12}$	4	0.3810	0.3357	L: X_FGCTAAAAGCATCTCCTTACC U: CGGCATAGAAATCTTTTACA	AF417693
Hl-8	99-121	$(GT)_{17}(T)_2(G)_7$	12	0.9048	0.8850	L: X_HCACAGGGATTAGGGGAAAG U: AGCCTTAAAAGTTGTCTAT	AF140585
Hl-14	230-236	$(GT)_{18}$	4	0.7143	0.7189	L: X_TGACCTGAGCTGAAGGCAGAC U: GTTTGTTCAGTGTGTCCATTGTAGTTAC	AF140586
Hl-15	119-139	$(GT)_{14}$	11	0.7143	0.8397	L: X_TCATCTTGTAGTGCCAAAAAC U: ATCTTTCAGTTGACCCTTCT	AF140587
Hl-16	134-140	$(GT)_{13}$	4	0.4286	0.3717	L: X_FCACTTATCTCGCCCTATATCCA U: CAGCCACAGCCAACACAA	AF140588
Hl-20	93-125	$(GT)_{20}$	10	0.8571	0.8722	L: X_FCTCAACACAGGCGTAATATTG U: GATCTTTGACAAGGAGAGTATGTT	AF140589
Lc-5	153-167	$(GT)_9$	9	0.8000	0.6873	L: X_TATCTTCAGGCTTTCTTCT U: TTCACGGACTCAAATAAT	AF417694
Lc-6	211-251	$(GT)_{16}C(GT)_7$	15	0.9200	0.8955	L: X_FGATCTTCACACAGACAC U: GCTATGCTTCCTCCA	AF140580
Lc-13	136-160	$(GT)_{17}$	13	0.8400	0.8980	L: X_HACAGCCTGTAATAATGTTGTG U: GTTTCTGGCCTTAATACAGCAAA	AF140581
Lc-18	275-302	$(A)_{15}(GT)_{11}$	19	0.8400	0.9282	L: X_TATTCTCCTCTCACCCCTG U: AATCGGCTGCTGGTAAAT	AF140582
Lc-26	285-303	$(GT)_6C(GT)_2A(GT)_4A(GT)_9$	7	0.8000	0.7886	L: X_FCTCAAGGGACTGAGCCACTCA U: ACGGCAGGATTCTGAAACACT	AF140583
Lc-28	128-136	$(GT)_{11}$	5	0.6800	0.6841	L: X_TTCATATAATACCCACCTCTGTAAG U: TGCCTCGTGATGAAAAACT	AF140584

Table 6-2. Results of cross-specific amplification using 24 microsatellite primer sets derived from the Antarctic Monachines. For the Weddell Seal, leopard seal, and crabeater seal size range is given in base pairs followed by the number of observed alleles in parentheses. Observed and expected heterozygosity follow, respectively, and are calculated from the Weddell Seal, leopard seal and crabeater seal populations described in Table 6-1. For the remaining species, size range in bp and number of observed alleles (in parentheses) found in five individuals are reported. Weddell seal *Leptonychotes weddellii;* Leopard seal *Hydrurga leptonyx*; Crabeater seal *Lobodon carcinophagus*; Ross seal *Ommatophoca rossii*; Northern Elephant seal *Mirounga angustirostris*; Bearded seal *Erignathus barbatus*; Hooded seal *Cystophora cristata*; Grey seal *Halichoerus grypus*; Harp seal *Phoca groenlandica*; Harbour seal, *Phoca vitulina*; Ringed seal *Phoca hispida*; New Zealand Fur seal, *Arctocephalus forsteri*; Walrus *Odobenus rosmarus*

	Weddell Seal	Leopard Seal	Crabeater Seal	Ross Seal	Northern Elephant Seal	Bearded Seal	Hooded Seal	Grey Seal	Harp Seal	Harbour Seal	Ringed Seal	New Zealand Fur Seal	Walrus
Lw-4		127-171 (10) (0.9048) (0.8722)	117-168 (20) (0.9600) (0.9355)	98-124 (3)	133 (1)	131-149 (6)							125-129 (3)
Lw-5		87-114 (11) (0.8571) (0.8990)	88-112 (16) (0.5600) (0.9110)	88-102 (5)									
Lw-7		155-171 (4) (0.5238) (0.4588)	153-173 (10) (0.8800) (0.7992)	156-158 (2)	151 (1)	155-159 (2)	157-173(4)	155-175 (3)	151-161 (6)	173-181 (4)	155-157 (2)	151 (1)	
Lw-8		103-105 (2) (0.3810) (0.4181)	107-131 (11) (0.9600) (0.8918)	103-111 (6)	107-109 (2)	115-119 (3)	101 (1)	97 (1)	101-107 (3)	103 (1)	97-105 (2)		
Lw-9		98-102 (3) (0.6190) (0.6272)	86-88 (2) (0.0400) (0.0400)	94-98 (3)							111-113 (2)		
Lw-10		94-132 (10) (0.9524) (0.9036)	92-122 (6) (0.7200) (0.7192)	106-130 (6)	118-122 (2)	122-138 (8)	107-115 (5)	107 (1)	106-118 (7)	109 (1)	105 (1)	116-132 (6)	112-114 (2)
Lw-11		165-179 (7) (0.7619) (0.8444)	147-177 (13) (0.8000) (0.9200)	163-173 (5)				173-179 (4)		169-177 (5)			
Lw-15		142-146 (3) (0.3810) (0.5669)	138-164 (12) (0.9600) (0.8898)	145-161 (6)	119 (1)	132-138 (2)	136-160 (4)	127-157 (2)	137-141 (3)	143 (1)	146-154 (4)		
Lw-16		161-175 (7) (0.5714) (0.6945)	145-206 (21) (0.9600) (0.9527)	160-190 (5)	163-165 (2)								
Lw-18*		106-116 (4) (0.5714) (0.7143)	114-140 (11/11) (0.7273) (0.9004)	112-144 (7)	103-107 (2)	110-120 (2)	90-96 (3)	124-136 (4)	102-110 (4)	108-118 (4)	124-134 (5)	110-112 (2)	
Lw-20		122-140 (6) (0.6190) (0.5581)	112-144 (17) (0.8800) (0.9412)		124-136 (3)	144-164 (3)	124 (1)	124-132 (2)	124 (1)	130-136 (4)	124 (1)		124 (1)
Hl-2	106-108 (2) (0.4792) (0.4991)		96-108 (5) (0.8000) (0.7061)	90-106 (2)	106 (1)	102-110 (4)	96-98 (2)	94 (1)	95-110 (6)	98-100 (2)	98-106 (4)		104 (1)
Hl-4	129-137 (7) (0.4792) (0.5177)		124-141 (10) (0.7200) (0.8408)	124-128 (3)	123 (1)	139-147 (4)	133-151 (5)	124-134 (3)	130-156 (5)	129 (1)	139-161 (6)	125-135 (3)	
Hl-8	97-123 (13) (0.8125) (0.8613)		95-123 (13) (0.9200) (0.9118)	90-100 (6)	97-101 (3)	109-123 (4)	103-117 (6)	105-111 (3)	99-119 (8)	95 (1)	97-117 (6)	85 (1)	95-99 (3)

Table 6-2. *Continued*

	Weddell Seal	Leopard Seal	Crabeater Seal	Ross Seal	Northern Elephant Seal	Bearded Seal	Hooded Seal	Grey Seal	Harp Seal	Harbour Seal	Ringed Seal	New Zealand Fur Seal	Walrus
Hl-14	216-246 (12) (0.8229) (0.8247)		212-248 (14) (0.9600) (0.9184)										
Hl-15	119-161 (13) (0.5521) (0.5579)		119-151 (13) (0.8000) (0.8759)	112-116 (3)	121 (1)	114 (1)	135-157 (7)	135-141 (3)	136-162 (8)	123-125 (2)	117-165 (7)	146 (1)	121 (1)
Hl-16	128-146 (7) (0.5938) (0.5992)		128-154 (14) (0.9200) (0.9127)	130-152 (4)	126 (1)	140-146 (4)	132-138 (4)	145-149 (3)	136-150 (5)	145-151 (3)	139-157 (6)	149-189 (7)	139-147 (5)
Hl-20	97-107 (6) (0.6146) (0.6384)		93-111 (8) (0.4400) (0.8441)	98-114 (6)	107(1)	103-111 (4)	107-117 (5)	107-117 (4)	97-115 (8)	107-113 (4)	109-123 (8)	107-119 (3)	107-119 (7)
Lc-5	161-165 (3) (0.0833) (0.0807)	157-163 (3) (0.5238) (0.5331)		162-164 (2)	158 (1)	161-171 (4)	164-174 (4)	157-181 (5)	161-183 (6)	167-169 (2)	161-185 (10)	157-163 (4)	
Lc-6	223-253 (13) (0.9167) (0.8922)	217-235 (3) (0.2381) (0.2218)		229-235 (4)	239 (1)	247-249 (2)		237-247 (3)	143-221 (8)	239-241 (2)	231-249 (6)		
Lc-13	132-158 (8) (0.6771) (0.7115)	128-164 (10) (0.8095) (0.8839)		131-143 (6)	140 (1)	156-188 (6)	142 (1)	142-144 (2)	139-141 (2)	142 (1)	142-146 (3)		140-154 (5)
Lc-18	278-283 (5) (0.6354) (0.6180)	278-294 (8) (0.8095) (0.7305)		285-311 (8)	274 (1)	280-286 (3)	289-297 (5)	300-309 (6)	287-306 (7)	287-294 (4)	198-310 (6)	293-301 (5)	
Lc-26	279-305 (11) (0.7917) (0.8491)	297-315 (7) (0.7619) (0.8200)		293-309 (7)	282 (1)	293 (1)	295-309 (8)	305-321 (3)	275-(8)	312-320 (5)	300-330 (8)	316-318 (2)	
Lc-28	127-135 (4) (0.3476) (0.3505)	131-145 (6) (0.7619) (0.7305)		126-130 (2)	134 (1)	152-160 (5)	130-148 (6)	136 (1)	136-158 (8)	146-150 (3)	134-156 (6)	134-154 (5)	149-153 (3)